# Genetic analyses of type 2 diabetes related metabolic risk factors : a twin study

# GENETIC ANALYSES OF TYPE 2 DIABETES RELATED METABOLIC RISK FACTORS: A TWIN STUDY

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# *GENETIC ANALYSES OF TYPE 2 DIABETES RELATED METABOLIC RISK FACTORS: A TWIN STUDY*

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***Diabetes mellitus – a geneticist's nightmare***

- James V Neel, 1976 -

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## ABBREVIATIONS

| | |
|---|---|
| A | Additive genetic effects |
| ABCC8 | ATP-binding cassette, subfamily C, member 8 or sulfonylurea receptor 1 |
| ADIPOQ | Adiponectin |
| AGTR1 | Angiotensin II type 1 receptor |
| AIC | Akaike's information criterion |
| Apo | Apolipoprotein |
| ASP | Affected sib-pair |
| BMI | Body mass index |
| C | Common environmental effects |
| CETP | Cholesteryl ester transfer protein. |
| CYP1A1 | Cytochrome P450 1A1 |
| D | Non-additive genetic effects |
| DA | Diamniotic |
| DC | Dichorionic |
| DSP | Discordant sib-pair |
| DZ | Dizygotic |
| DZF | Dizygotic women |
| DZM | Dizygotic men |
| DZOS | Dizygotic opposite sex |
| E | Unique environmental effects |
| EFPTS | East Flanders Prospective Twin Survey |
| ENPP1 | Ectoenzyme nucleotide pyrophosphate phosphodiesterase 1 |
| FAAH | Fatty acid amide hydrolase |
| FPG | Fasting plasma glucose |
| GABA | γ-aminobutyric acid |
| GAD2 | Glutamate decarboxylase 2 |
| GCK | Glucokinase |
| GWA | Genome wide association |
| HDL | High-density lipoprotein |
| HL | Hepatic triglyceride lipase |
| HOMA | Homeostasis model assessment |
| IBD | Identical by descent |
| IDL | Intermediate density lipoprotein |
| IFG | Impaired fasting glucose |
| IGF1 | Insulin-like growth factor 1 |
| IGF2 | Insulin-like growth factor 2 |
| IGF1R | Insulin-like growth factor 1 receptor |
| IGF2R | Insulin-like growth factor 2 receptor |
| IGFBP1 | Insulin-like growth factor binding protein 1 |
| IGT | Impaired glucose tolerance |
| IL6 | Interleukin 6 |
| INSR | Insulin receptor |
| IRS1 | Insulin receptor substrate 1 |
| IRS2 | Insulin receptor substrate 2 |

| | |
|---|---|
| KCNJ11 | Potassium inwardly-rectifying channel, subfamily J, member 11 |
| LD | Linkage disequilibrium |
| LDL | Low-density lipoprotein |
| LEP | Leptin |
| LEPR | Leptin receptor |
| LOD | Logarithm of the odds |
| LPL | Lipoprotein lipase |
| MA | Monoamniotic |
| MC | Monochorionic |
| MODY | Maturity-onset diabetes of the young |
| mtDNA | Mitochondrial DNA |
| MZ | Monozygotic |
| MZF | Monozygotic women |
| MZM | Monozygotic men |
| NEFA | Non-esterified fatty acids |
| NPY | Neuropeptide Y |
| OGTT | Oral glucose tolerance test |
| PCFA | Principal component factor analysis |
| PKCθ | Protein kinase C-θ |
| PPARγ | Peroxisome proliferator activated receptor-γ |
| PYY | Peptide YY |
| QTL | Quantitative trait loci |
| RETN | Resistin |
| RFLP | Restriction fragment length polymorphism |
| S4SF | Sum of four skinfold thicknesses |
| SEM | Structural equation modelling |
| SNP | Single nucleotide polymorphism |
| T1D | Type 1 diabetes |
| T2D | Type 2 diabetes |
| TDT | Transmission disequilibrium test |
| TNFα | Tumour necrosis factor α |
| UCP2 | Uncoupling protein 2 |
| UTR | Untranslated region |
| VLDL | Very low-density lipoprotein |
| WHO | World Health Organization |
| WHR | Waist-to-hip ratio |

# CHAPTER 1

# GENERAL INTRODUCTION

This thesis describes a twin study in which a candidate gene approach is used to identify genetic variants underlying quantitative traits that predispose to or are associated with type 2 diabetes (T2D). In this chapter, metabolic abnormalities that induce or are associated with T2D are briefly discussed. Subsequently, the underlying principles of the different genetic methodologies used and the candidate genes studied in this thesis are introduced. At the end of this chapter the aims of this thesis are presented.

## 1.1 DIABETES

### *1.1.1 Prevalence of diabetes*

Diabetes mellitus or simply diabetes defines a group of metabolic disorders characterised by high blood glucose levels (hyperglycaemia) due to insufficient insulin secretion, action or both.[1] The worldwide incidence of diabetes is increasing at an alarming rate. In 2007 the number of people in the USA with diagnosed diabetes reached 17.5 million and the disease has become one of the leading causes of death in the USA.[3] In the Netherlands, about 700,000 (4.4%) people above the age of 20 suffered from diabetes in the period 2004-2006, representing a nearly twofold increase compared to the early 1990s, which was mainly due to a large increase in the number of older, obese diabetes patients (see Figure 1).[4] In 2003, about 900,000 persons in the Netherlands above the age of 60 had pre-stage diabetes (impaired glucose tolerance) and it was estimated that another 115,000 to 300,000 persons were unaware of having diabetes.[5] Moreover, at least five million (30%) Dutch people were at increased risk for diabetes due to overweight and/or an inactive lifestyle.[5]

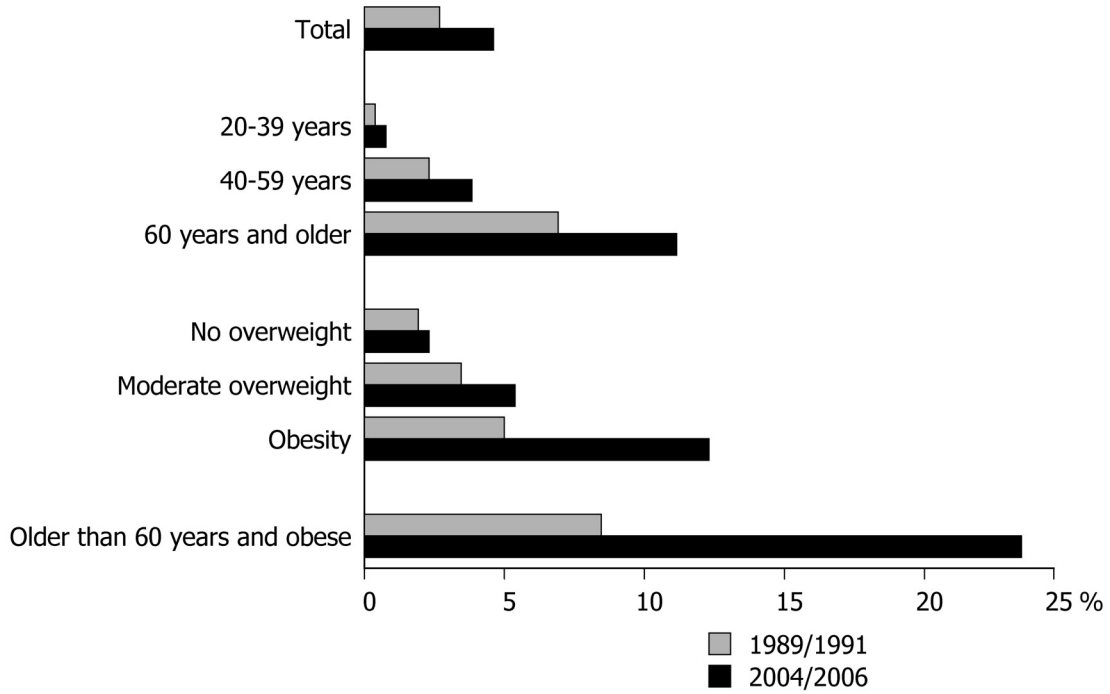


**Figure 1.** Total proportion of the Dutch population above the age of 20 suffering from diabetes (includes all types of diabetes) in the periods 1989-1991 and 2004-2006; subdivided by age and obesity status (adapted from reference [4]). No overweight is defined as body mass index (BMI) ≤ 25, moderate overweight is defined as BMI between 25 and 30, and obesity is defined as BMI > 30.

Diabetes is associated with microvascular (retinopathy, nephropathy and neuropathy) and macrovascular (coronary, cerebrovascular and peripheral arterial disease) complications.[6,7] Worldwide, diabetic retinopathy explains 5% of all blind people and diabetic nephropathy is the most common cause for renal replacement therapy. In addition, diabetes is the most common reason of non-traumatic amputation.[7] Macrovascular complications (atherosclerosis) account for approximately 80% of all mortality in diabetic subjects, with about 75% due to coronary arterial disease and 25% to cerebral or peripheral arterial disease.[8] Due to diabetes life expectancy decreases with 5-10 years.[7] These complications not only decrease the quality of life of the patients, but also result in a huge financial pressure on health care systems. For instance, the total costs of diabetes in the USA in 2007 have been estimated to be $174 billion. This implies that one in ten health care dollars spent in the USA is attributed to diabetes and its complications, demonstrating that diabetes places an enormous burden on society.[3,9]

### *1.1.2 Classification of diabetes*

Diabetes is the final result of distinct pathogenic processes and it can be roughly divided into four different types[1]:

*Type 1 diabetes*

Type 1 diabetes (T1D) also called insulin-dependent diabetes is mainly caused by autoimmune destruction of the insulin-producing pancreatic ß-cells. T1D is characterised by the presence of autoantibodies against ß-cells antigens and usually leads to absolute insulin deficiency. T1D accounts for approximately 5-10% of all diagnosed diabetes patients and it typically affects young people, given that most of the cases are diagnosed before the age of 18.[1,10] Up to 50% of the familial clustering of T1D is explained by protective and predisposing haplotypes at the human leukocyte antigen (HLA) locus, encoding cell-surface antigen-presenting proteins. The remainder is contributed by multiple loci.[11,12] Although genetic factors are important determinants of T1D susceptibility, the concordance rate of T1D in monozygotic (MZ) twins is only 50% compared to 10% in dizygotic (DZ) twins.[13] This indicates that genetic susceptibility alone is not sufficient to develop T1D, and it is suggested that environmental factors like diet and viral infections trigger β-cell autoimmunity in genetically predisposed individuals.[14]

*Gestational diabetes*

Gestational diabetes is defined as any degree of glucose intolerance with onset or first recognition during pregnancy.[1] In the USA about 4% of all pregnancies are complicated by gestational diabetes and this condition is associated with high fetal birth weight and an increased risk for caesarean delivery.[1,15] In addition, both the mother with gestational diabetes and the offspring are at increased risk for developing diabetes, usually type 2, later in life.[16-18]

*Other types of diabetes*

This category contains rare monogenetic defects causing ß-cell dysfunctioning (maturity-onset diabetes of the young (MODY), mtDNA mutations), rare monogenetic defects resulting in impaired insulin action, diseases of the exocrine pancreas that cause diabetes, endocrinopathies (disorders characterised by excess amounts of hormones that antagonise insulin action can cause diabetes), drug- or chemical-induced diabetes, infections, uncommon forms of immune-mediated diabetes and other genetic syndromes associated with an increased incidence of diabetes.[1]

*Type 2 diabetes*

In this thesis we focus on the most common form of diabetes, type 2 diabetes (T2D) also called non-insulin-dependent diabetes, accounting for approximately 90-95% of all diabetes patients.[1] In contrast to T1D, T2D is not caused by autoimmune destruction of the pancreatic ß-cells, but is the result of deficiency in both insulin action (insulin resistance) and secretion. At least initially, and often throughout life, T2D patients do not need insulin therapy.[1] T2D is associated with the western lifestyle and for that reason the rapid worldwide increase in the incidence of diabetes is mainly attributed to the increase in patients with T2D.[19] In the following section, metabolic abnormalities that are believed to induce or are associated with T2D are discussed.

### *1.1.3 T2D and related metabolic abnormalities*

*Insulin resistance*

In response to rising glucose levels, insulin is secreted by the pancreatic ß-cells (see Box 1) and acts through binding to the insulin receptor or to the insulin-like growth factor 1 receptor. This initiates phosphorylation of intracellular substrates, including the insulin receptor substrate proteins (IRS1-6), which are connected to the activation of two main downstream signalling pathways. The first pathway is the phosphatidylinositol-3-kinase (PI3K)-AKT/protein kinase B (PKB) pathway that is largely responsible for the metabolic actions of insulin. The second pathway is the Ras-mitogen activated protein kinase (MAPK) pathway which regulates gene expression, but also interacts with the PI3K pathway to control cell growth and differentiation.[20] Insulin plays a key role in the maintenance of glucose homeostasis. It regulates blood glucose by promoting peripheral (primary muscle)

***Box 1: Insulin secretion***

After a meal, glucose is taken up into the pancreatic ß-cells in the islets of Langerhans through the glucose transporter isotype 2 (GLUT2) and gets phosphorylated into glucose-6-phosphate by glucokinase. In the glycolytic pathway, glucose-6-phosphate is converted into pyruvate, which is transported into the mitochondria and degraded into acetyl-CoA. Subsequently, acetyl-CoA enters the citric acid cycle where it is oxidised further, yielding $CO_2$ and the reduced coenzymes nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide ($FADH_2$). These reduced coenzymes then enter the electron transport chain, which generates ATP through oxidative phosphorylation. ATP synthesised in the mitochondrial matrix is transported into the cytosol, resulting in an increased cytosolic ATP/ADP ratio and closure of the ATP-sensitive potassium ($K_{ATP}$) channel. The closure is followed by membrane depolarisation, which activates voltage gated calcium channels and leads to intracellular $Ca^{2+}$ rise triggering insulin secretion.[2]

and splanchnic (liver) glucose uptake, suppressing hepatic glucose production (by decreasing gluconeogenesis and glycogenolysis) and stimulating glycogen synthesis (glycogenesis) in the liver and skeletal muscle. In addition, when the liver is saturated with glycogen (approximately 5% of liver mass), further hepatic glucose uptake will be used for hepatic fatty acid synthesis (lipogenesis) that is also stimulated by insulin. Furthermore, in adipose tissue, insulin inhibits the breakdown of triglycerides into glycerol and fatty acids (lipolysis).[21-24]

In the presence of insulin resistance, the body does not respond sufficiently to normal circulating insulin levels. To compensate for this reduced efficiency of insulin action, pancreatic ß-cells increase insulin release to maintain normoglycaemia. This process called β-cell compensation is not completely understood, but both enhanced β-cell function and expansion of the β-cell mass are thought to be involved.[25] The increase in β-cell mass is probably due to an increase in β-cell number, but hypertrophy might also contribute.[26] However, when insulin resistance is accompanied by ß-cell dysfunction, then ß-cells are unable to compensate for the increased demand for insulin. This results in insufficient suppression of hepatic glucose production and decreased efficiency of muscle, adipose tissue and liver glucose uptake. In addition, non-esterified fatty acids (NEFA) levels will rise due to a decreased lipolysis suppression in adipose tissue (Figure 2). ß-cell dysfunction leads to impaired glucose tolerance, impaired fasting glucose and at the extreme T2D (see Box 2).[20]

***Box 2: Diagnostic criteria for diabetes***

Based on fasting plasma glucose (FPG)[1]

- Normal fasting glucose (NFG): FPG < 100 mg/dl or < 5.6 mmol/l
- Impaired fasting glucose (IFG): FPG 100–125 mg/dl or 5.6–6.9 mmol/l
- Diabetes: FPG ≥ 126 mg/dl or ≥ 7.0 mmol/l

Based on 2-h glucose values of an oral glucose tolerance test (OGTT)[1]

- Normal glucose tolerance (NGT): 2-h glucose < 140 mg/dl or < 7.8 mmol/l
- Impaired glucose tolerance (IGT): 2-h glucose 140–199 mg/dl or 7.8–11.1 mmol/l
- Diabetes: 2-h glucose ≥ 200 mg/dl or ≥ 11.1 mmol/l

*Obesity*

The worldwide increase in the prevalence of T2D is strongly correlated with the dramatically rising incidence of obesity. The World Health Organisation (WHO) estimated that in 2005, approximately 1.6 billion adults (> 15 years) were overweight (BMI ≥ 25) and at least 400 million were obese (BMI ≥ 30). It is expected that in 2015 approximately 2.3 billion adults will be overweight and more than 700 million will be obese.[27]

Obesity is characterised by excess accumulation of adipose tissue. Adipose tissue, in the past considered as a passive energy store of triglycerides, is now regarded as an active endocrine organ that modulates metabolism by secreting a variety of active molecules, including proinflammatory cytokines, NEFA and hormones. In obese individuals, the production of many of these molecules is increased, including the production of proinflammatory cytokines associated with a chronic low-grade inflammatory state.[20] This obesity-associated chronic inflammation is thought to induce insulin resistance. For instance the proinflammatory cytokine tumour necrosis factor α (TNFα), which is over-expressed in adipose tissue of obese subjects,[28] can phosphorylate IRS1 on serine, thereby blocking its tyrosine phosphorylation

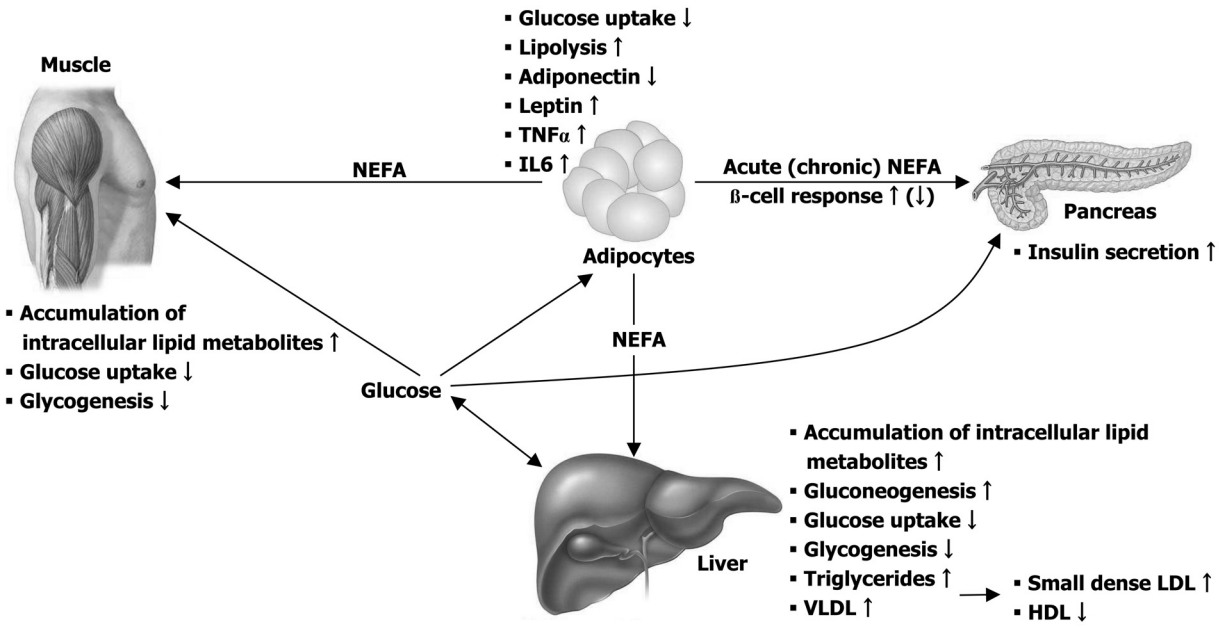


**Figure 2.** Pathophysiology of insulin resistance.

and downstream activation of the insulin signalling cascade. TNFα is also able to inhibit insulin signalling independent of IRS1, by reducing adipocyte expression of genes that contribute to glucose homeostasis, including the insulin receptor and glucose transporter 4 (GLUT4).[29] Circulating levels of the well-known proinflammatory cytokine interleukin-6 (IL6) are also elevated in obesity, but its biological effect on insulin sensitivity is still disputed.[30,31]
Adipose tissue in obese individuals is characterised by hypertrophic adipocytes that are less sensitive to insulin and have a higher lipolytic rate.[32] This results in increased serum NEFA levels, which is considered to be an important link between obesity and insulin resistance and T2D. In the pancreatic ß-cells, short term elevation of NEFA plasma levels stimulates insulin secretion, however, chronic elevation of NEFA levels is thought to result in an impaired ß-cell response.[33] Increased NEFA levels also leads to ectopic fat accumulation in nonadipose tissues such as liver and muscle, that appears to induce insulin resistance in these tissues. In muscle, increased accumulation of intracellular lipid metabolites (e.g. diacyglycerol) can activate the serine-threonine kinase, protein kinase C-θ (PKCθ) that phosphorylates IRS1 on serine and thereby inhibiting insulin action.[29,33,34] In the liver, a similar mechanism of NEFA-induced insulin resistance is believed to occur, where accumulation of intracellular lipid metabolites can activate a serine kinase cascade involving PKCε. This results in a decreased tyrosine phosphorylation of IRS2, which is a key mediator of hepatic insulin action.[35,36] Consequently, elevated NEFA levels can result in reduced hepatic glycogenesis and increased hepatic gluconeogenesis (Figure 2).[33,35,36]
Adipose tissue is also the major source of circulating leptin, a hormone that regulates energy homeostasis by suppressing food intake and promoting energy expenditure by acting on the hypothalamus. Human subjects with total congenital leptin deficiency or a leptin receptor deficiency are extremely obese. However, the majority of obese people have high circulating leptin levels, indicating leptin resistance.[37,38] Another adipocyte-derived hormone adiponectin is believed to play a role in obesity-induced insulin resistance. In contrast to the other currently known adipocyte-derived hormones, circulating levels of adiponectin are low in obese people. In addition, increased adiponectin levels are associated with improved glucose

tolerance and are negatively correlated with cardiovascular risk factors. Adiponectin administration in rodents resulted in improved glucose tolerance without stimulating insulin release and demonstrated anti-atherosclerotic effects.[39,40]
As outlined above, obesity is an important modulator of insulin sensitivity, however, the type of body fat distribution seems to be more important than general obesity itself: a central or abdominal fat distribution is considered to have more adverse metabolic effects than a peripheral fat distribution.[41] Lean individuals with a peripheral fat distribution have a higher insulin sensitivity compared to lean individuals with a central fat distribution. Especially, intra-abdominal or visceral adipose tissue is believed to modulate insulin sensitivity, which might be due to the enhanced lipolytic activity of visceral fat cells resulting in an increased NEFA flux to the liver. However, there is also evidence indicating that visceral adipose tissue produces excessive amounts of proinflammatory cytokines and low amounts of the insulin-sensitising hormone adiponectin.[20,33]

*Dyslipidaemia*

Insulin resistance, obesity and T2D are accompanied with an abnormal lipid profile including elevated levels of the very low-density lipoprotein (VLDL) (Figure 2).[8,42,43] The apolipoproteinB (apoB) containing VLDL is a triglyceride-rich lipoprotein that transports triglycerides from the liver to peripheral tissues, including muscle and adipose tissue. In the capillary beds of these tissues, triglycerides are removed from mature VLDL by lipoprotein lipase (LPL) leading to fatty acid uptake by myocytes and adipocytes. Lipolysis of VLDL by LPL results in a reduction of the triglycerides content of these particles and leads to the formation of the more cholesterol rich intermediate density lipoprotein (IDL). In the circulation, IDL donates borrowed surface phospholipids, apoC and apoE to high density lipoproteins (HDL), and exchanges a part of their triglyceride content to HDL in return for cholesteryl ester. The latter exchange is facilitated by cholesteryl ester transfer protein (CETP). IDL particles can be cleared by the liver or can be converted into the cholesterol-rich low-density lipoprotein (LDL) by further extraction of nearly all remaining triglycerides by hepatic triglyceride lipase (HL).[44] LDL can also be produced directly by the liver.[8] Subsequently, LDL can be removed by the liver or taken up by nonhepatic tissues via the LDL receptor or through non-receptor mediated LDL uptake.[8,44,45]
It is believed that the enhanced NEFA flux to the liver, due to the increased lipolytic activity of adipose tissue and/or decreased adipocyte NEFA uptake, is responsible for the increased VLDL secretion in insulin resistance.[8,42] In addition, it has also been described that de novo lipogenesis might be increased in hyperinsulinemic individuals, and that the rate of apoB degradation is decreased in insulin resistance.[8,45,46] Moreover, the clearance of triglycerides-rich lipoproteins may be reduced due to a decreased activity and expression of lipoprotein lipase and the LDL-receptor.[8,45,47] Plasma triglycerides levels are also elevated in insulin resistance, obesity and T2D, which is mainly due to the increase in VLDL.[42,45] Both elevated triglycerides and VLDL levels are associated with an increased cardiovascular risk.[48]
LDL-cholesterol levels, that represent a major cardiovascular risk factor, are normal (male) or only modestly increased (female) in T2D and insulin resistance.[8] However, there is a shift towards the more atherogenic small dense LDL particles, which might be the result of increased activity from HL and CETP in insulin resistance. CETP transfers triglycerides from VLDL toward LDL in change for cholesteryl ester resulting in triglyceride-rich LDL particles.

These particles are rapidly lipolysed by HL leading to small dense LDL particles.[8,42,45] Small dense LDL particles have an increased susceptibility to oxidation and oxidised LDL is rapidly taken up through macrophages, leading to the formation of foam cells that are characteristic components of atherosclerotic plaques.[42,45]
Insulin resistance, obesity and T2D are also associated with low HDL-cholesterol levels.[8,42] ApoA-containing HDL, secreted by the liver and intestine, that obtained phospholipids, apoC and apoE from triglyceride-rich lipoproteins (e.g. IDL), removes excess cholesterol and phospholipids from nonhepatic cells by binding to the ATP binding cassette transporter type 1 (ABCA1) transporter. Subsequently, the cholesterol gets esterified by the enzyme lecithin cholesterol acyltransferase (LCAT), leading to the formation of a larger spherical HDL particle. "Full" HDL returns to the bloodstream where it transfers apoC and apoE, and exchanges cholesteryl ester for triglycerides with triglyceride-rich lipoproteins.[45] In the liver, HDL can be cleared by binding to the scavenger receptor class B type 1 (SRB1) that results in uptake of the cholesterol ester content without degradation of the HDL particle (selective lipid uptake). Alternatively, HDL can be cleared by endocytic uptake in the liver and the kidney resulting in degradation of the whole particle (holoparticle uptake).[49] The main function of HDL is transport of excess cholesterol from the nonhepatic tissues to the liver for excretion in the bile. In addition, HDL is involved in the metabolism of triglyceride-rich lipoproteins, and has antioxidant and anti-inflammatory effects in the arterial wall. Hence HDL-cholesterol is protective against cardiovascular disease.[44,50,51]
In insulin resistance, HDL-cholesterol levels are decreased due to an increased catabolism of HDL particles.[45] The major cause for this augmented HDL catabolism is the increased concentration of triglyceride-rich lipoproteins. Due to enhanced levels of triglyceride-rich lipoproteins, the CETP facilitated transfer of triglycerides from triglyceride-rich lipoproteins to HDL is increased. This results in enhanced levels of triglyceride-rich HDL particles that are a very good substrate for HL of which activity is increased in insulin resistance, resulting in an enhanced HDL catabolism.[8,45,49]

### *Low birth weight*

About 20 years ago, Barker and colleagues observed that low birth weight was associated with an increased risk for adult metabolic disorders like cardiovascular disease and T2D.[52-54] In order to explain their observation Hales and Barker[55] proposed the 'thrifty phenotype hypothesis' and postulated that an adverse intra-uterine environment leads to permanent changes of the structure and function of certain organs and tissues. These adaptations would result in an altered metabolic state considered to be beneficial for survival under circumstances of insufficient nutrient supply, but unfavourable in post-natal life with abundant nutrient supply.[55] Genetic factors were not considered in this hypothesis, and therefore a second hypothesis, the 'fetal insulin hypothesis', was put forward.[56] This hypothesis postulates that birth weight and T2D have common genetic antecedents, and that this genetically determined insulin resistance would result in low insulin-mediated fetal growth as well as in insulin resistance in adulthood.[56]
Many studies have confirmed the association between low birth weight and increased risk of adult metabolic diseases.[57] In addition, the human data is strongly supported by experiments in animals, where dietary restriction or surgical interventions inducing fetal growth restriction resulted in offspring having adverse adult metabolic phenotypes.[58] Consequently, low birth

weight has grown an accepted risk factor for T2D, however it remains to be clarified to what extent environmental or genetic factors explain the underlying mechanism.[59]

### *1.1.4 Genetics of T2D*

Epidemiological studies have demonstrated that factors related to the western lifestyle such as high calorie diets, high amounts of saturated fat intake, physical inactivity, urbanisation, mechanisation, smoking and alcohol consumption, are strongly associated with T2D.[60] Besides the key role of these lifestyle factors, there is also clear evidence that genetic susceptibility contributes to the development of T2D.
Indications for a strong genetic component in T2D development are well established by family studies, in which a positive family history conferred 2-4 fold increased risk for T2D.[61] Further indications have come from studies in specific ethnic groups. For instance within the multiracial USA population, Pima Indians of Arizona have the highest prevalence of T2D,[62] and the risk for Mexican-Americans and non-Hispanic blacks is almost twice that of non-Hispanic whites.[63] Furthermore, the concordance rate for T2D of monozygotic (MZ) twins is much higher compared to dizygotic (DZ) twins.[64,65]
In order to get a better understanding of the pathogenesis of T2D a lot of research has been focused on the genetic component of the disease. However, because of the polygenic character of the disease and the modest effect size of individual genes, it remains a tremendously difficult task to identify the underlying genetic factors. Consequently, the T2D genes that have been identified up till now only explain a small proportion of the excess familial risk.[66,67]

## 1.2 HERITABILITIES AND TWINS

### *1.2.1 Heritabilities*

In order to unravel the genetics of human disease, it is of great interest to assess the size of the genetic component contributing to the disease or quantitative trait being studied. According to the biometrical genetics theory,[68] the total variance of a quantitative trait (P) can be decomposed into variances due to:

- additive genetic effects (A), which represents the sum of effects of the individual alleles at all loci influencing the trait,
- non-additive genetic effects (D), representing interactions between alleles at the same locus (dominance) or on different loci (epistasis),
- common environmental effects (C), environmental influences shared by family members,
- and unique environmental effects (E), environmental influences unique to an individual.[69]

Thus $P = A + D + C + E$

A commonly used measure to quantify to which extent genetic factors explain the total variance of a trait is the heritability. We can distinguish between the broad sense heritability ($H^2$) and the narrow sense heritability ($h^2$). The broad sense heritability estimates to which

extent the total variation of a trait can be explained by genetic variation ($H^2$ = (A + D)/P). And the narrow sense heritability ($h^2$) measures to which extent the total variation of a trait can be explained by additive genetic variation ($h^2$ = (A/P)). Heritability estimates can vary between 0 and 100% and they are specific for a particular population at a specific point in time; therefore they cannot be generalised to other populations. Since the heritability is an important determinant of the power to detect disease-related genes, high heritability estimates are preferred.[70]

To estimate heritabilities data of related individuals are required. For instance, family data can be used and significant correlations between family members strongly suggest that genetic factors are involved. However, besides their genetic material, family members also share environmental factors (diet, exercise etc) that may contribute to a higher similarity among them. To estimate the size of the underlying genetic component properly, it is essential to disentangle these shared environmental and shared genetic factors. This can be achieved by using adoption data, where shared environmental factors are considered to be unlikely when adoption occurred early in life. Significant correlations between biological parents and adopted children are a result of shared genetic factors, and significant correlations between adoptive relatives are due to shared environmental factors. Nevertheless, adoption data are difficult to obtain and might be biased because of selective placement.[69]

### *1.2.2 The classical twin study*

The classical twin study avoids the limitations described above and allows to split up the total variation of a trait into genetic, common environmental and unique environmental variance components. The method makes use of the fact that MZ twins are genetically identical and that DZ twins share 50% of their genetic material. Assuming that MZ and DZ twins share their common environment to the same extent, MZ and DZ twins have different correlations for the genetic variance components A and D, but the same correlations for the environmental variance components C and E. For MZ twins the correlation for both A and D is 1, and for DZ twins the correlations for A and D are ½ and ¼, respectively. In addition, for both MZ and DZ twins, the correlations for C and E are 1 and 0, respectively (Figure 3).[69,71]

A higher intra-pair correlation in MZ twins ($r_{MZ}$) compared to DZ twins ($r_{DZ}$) indicates that genetic factors are involved. In contrast, a $r_{DZ}$ greater than half of $r_{MZ}$ indicates that common environmental factors are important, and when both $r_{MZ}$ and $r_{DZ}$ are low then the total variation of a trait is mainly explained by environmental factors (Figure 4). Since non-additive genetic factors correlate perfectly in MZ twins but only for 25% in DZ twins, a $r_{DZ}$ less then half of $r_{MZ}$ suggests that non-additive genetic effects might be significant.[69] Traditionally, Falconer's formula has been used to estimate the heritability of a trait: $h^2 = a^2 = 2(r_{MZ} - r_{DZ})$.[72] In addition, because differences between MZ twins are only due to unique environmental effects, the intra-pair correlation of MZ twins can be used to estimate the contribution of unique environmental effects: $e^2 = 1 - r_{MZ}$. Subsequently, the common environmental component is the difference between the intra-pair correlations of MZ twins and the narrow sense heritability ($c^2 = r_{MZ} - h^2$).[69,72]

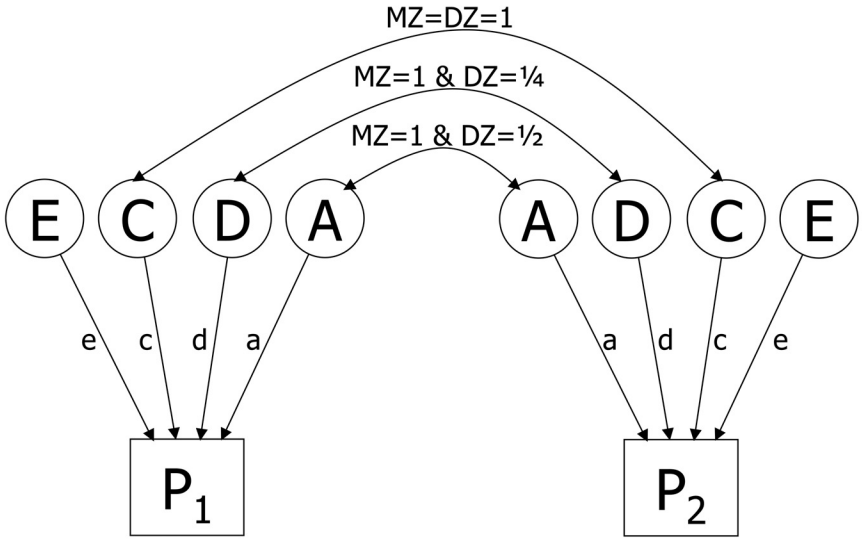


**Figure 3.** Path diagram for the basic univariate twin model (adapted from Rijsdijk and Sham[69]). Observed phenotypes ($P_1$ and $P_2$) for the first and the second twin are shown in rectangles, while latent factors (A, D, C and E) are shown in circles. Path coefficients of observed variables on the different latent factors are shown in lower case: a = additive genetic effect, d = non-additive genetic effect, c = common environmental effect, e = unique environmental effect. The additive genetic correlation is 1 for MZ and ½ for DZ twins. The non-additive genetic correlation is 1 for MZ and ¼ for DZ twins. Common environmental correlation is 1 for both MZ and DZ twins.

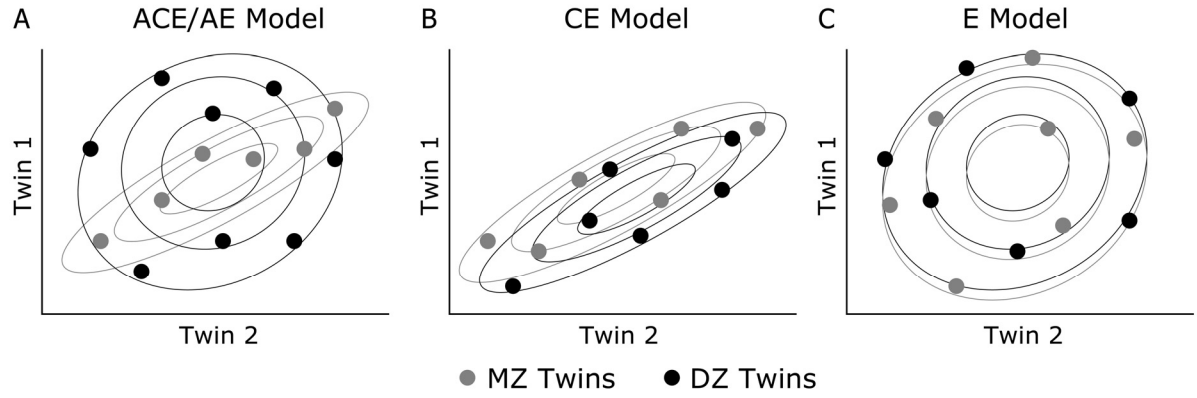


**Figure 4.** Scatter plots including both MZ and DZ twins. **A.** Higher concordance rate in MZ twins compared to DZ twins reflects genetic effects (ACE/AE model). **B.** Concordance rate in MZ twins similar to DZ twins reflects common environmental effects (CE model). **C.** Concordance rate low in both MZ and DZ twins reflects unique environmental effects (E model).

However, the approach described above does not supply information on how well a theoretical model fits to the data, nor does it enable testing different hypotheses. By using structural equation modelling (SEM), model parameters are estimated by minimising a goodness-of-fit statistic between the observed and expected variance-covariance matrices. In addition, SEM tests the fit of the hypothesised model, compares the fit of alternative models and provides confidence intervals for the parameter estimates. The expected variance–covariance matrices for MZ and DZ twins are defined as:

$$\textbf{ACE}\quad MZ\ \begin{matrix} & T1 & T2 \\ T1 & a^2 + c^2 + e^2 & a^2 + c^2 \\ T2 & a^2 + c^2 & a^2 + c^2 + e^2 \end{matrix} \qquad DZ\ \begin{matrix} & T1 & T2 \\ T1 & a^2 + c^2 + e^2 & \tfrac{1}{2}a^2 + c^2 \\ T2 & \tfrac{1}{2}a^2 + c^2 & a^2 + c^2 + e^2 \end{matrix}$$

$$\textbf{ADE}\quad MZ\ \begin{matrix} & T1 & T2 \\ T1 & a^2 + d^2 + e^2 & a^2 + d^2 \\ T2 & a^2 + d^2 & a^2 + d^2 + e^2 \end{matrix} \qquad DZ\ \begin{matrix} & T1 & T2 \\ T1 & a^2 + d^2 + e^2 & \tfrac{1}{2}a^2 + \tfrac{1}{4}d^2 \\ T2 & \tfrac{1}{2}a^2 + \tfrac{1}{4}d^2 & a^2 + d^2 + e^2 \end{matrix}$$

where $a^2$ = additive genetic variance, $c^2$ = common environmental variance, $d^2$ = non-additive genetic variance and $e^2$ = unique common environmental variance.
In the classical twin study, which includes data of MZ and DZ twins reared together, non-additive genetic and common environmental effects are confounded and cannot be estimated simultaneously. Therefore ACE and ADE models must be fitted separately. The significance of the variance components A, C or D in the model can be tested by dropping these parameters and comparing the fit of the models.[69]

### *1.2.3 Different type of MZ twins*

As already indicated in the previous section, DZ twins share 50% of their genetic material since they result from the ovulation and fertilisation of two oocytes (Figure 5). MZ twins are considered to be genetically identical because they originate from one zygote that splits in early embryonic development. However, the moment of splitting has implications for the intra-uterine development of MZ twins, and accordingly four subtypes of MZ twins can be distinguished (Figure 5):

- MZ dichorionic (DC) twins originate when splitting occurs before the 4$^{th}$ day post fertilisation. These twin pairs (about 34% of all MZ twins) have two amniotic membranes, two chorionic membranes and two placentas that may or may not be fused.
- MZ monochorionic diamniotic (MC DA) twins arise when the splitting events happens between the 4$^{th}$ and the 8$^{th}$ day post fertilisation. These twin pairs (about 64% of all MZ twins) have two amniotic membranes, one chorionic membrane and one placenta.
- MZ monochorionic monoamniotic (MC MA) twins arise when the splitting events happens after the 8$^{th}$ day post fertilisation. These twin pairs (~2%) have one amniotic membrane, one chorionic membrane and one placenta (Figure 5).[73]
- When the splitting events happens after the 12$^{th}$ day post fertilisation conjoined twinning can occur, of which the prevalence is estimated to range from 1:14,000 to 1:200,000 births.[74,75]

The classical twin study assumes an equal prenatal and postnatal environment for MZ MC, MZ DC and DZ twins. However, it has been demonstrated that intra-pair birth weight differences of MZ MC pairs are larger compared to MZ DC pairs, which results in a significantly lower intra-pair correlation for MZ MC compared to MZ DC pairs ($r_{MZMC} < r_{MZDC}$).[76,77] This clearly demonstrates that MZ MC twins suffer a more adverse intrauterine environment than MZ DC twins, which is possibly due to vascular anastomoses, unequal sharing of the placenta and/or greater competition for nutrient supply.[77] Because, the classical twin study does not discriminate between MZ MC and MZ DC twins, this method is

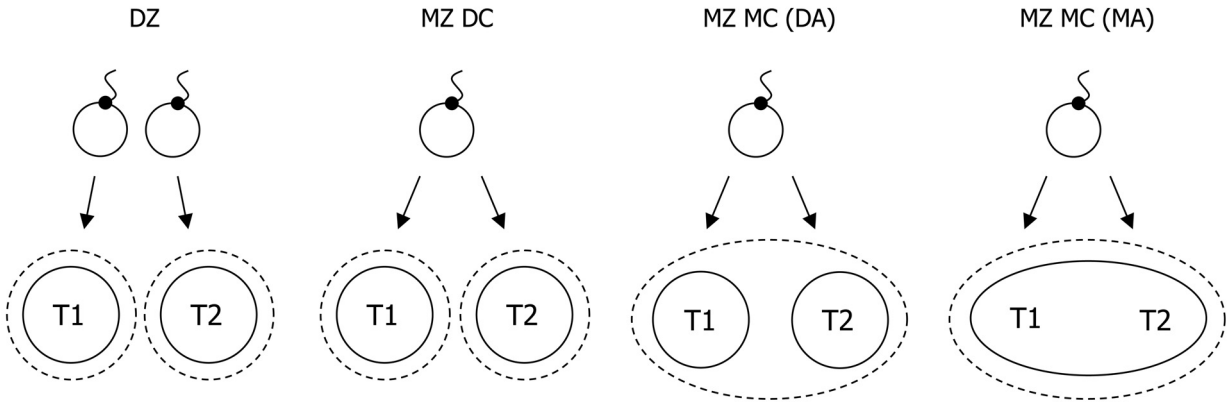


**Figure 5.** Type of twins according to zygosity, chorionic and amniotic membranes (adapted from reference [78]). DZ = dizygotic, MZ DC = monozygotic dichorionic, MZ MC (DA) = monozygotic monochorionic (diamniotic), MZ MC (MA) = monozygotic monochorionic (monoamniotic).

not suitable to estimate the heritability for birth weight. Since an adverse prenatal environment is also associated with adult metabolic disorders like T2D and cardiovascular disease,[57,79,80] it has been argued that the classical twin study is an unreliable method to estimate heritabilities for metabolic traits related to T2D and cardiovascular disease.[81]

## 1.3 LINKAGE STUDIES

In order to identify genetic variants underlying a trait or disease of interest statistical methods play a key role. A commonly used statistical method is linkage analysis, which tests within families for co-segregation of a random marker locus with the disease or trait of interest. There are two methods: 1) parametric or model-based linkage analysis where a specific disease model is used to describe segregation of the trait locus, or 2) non-parametric or model-free linkage analysis which does not require to specify a disease model. The underlying principles of these methods are outlined in the following sections.

### *1.3.1 Parametric linkage analysis*

If a disease locus and a marker locus are located on different chromosomes, then these two loci are independently transmitted to the next generation and are called unlinked. Consequently, the probability that two loci located on different chromosomes are transmitted together is 50%. In addition, if a disease locus and a marker locus are located far apart from each other on the same chromosome, then recombination as a result of crossing-over is very likely to occur and these loci still segregate independently. Conversely, if a marker locus is located near a disease locus on the same chromosome, they are more likely to segregate together and are called linked. Accordingly, the probability that these two loci segregate together is <50%. In the parametric linkage analysis the main quantity of interest is the recombination fraction ($\theta$) which represents the proportion of recombinant offspring and is a measure of the distance between a marker locus and the disease locus. When two loci are unlinked then $\theta=0.5$, and when two loci are linked $\theta<0.5$. Thus if $\theta=0$, then the marker locus and the disease locus are completely linked.[82]

The parametric linkage approach has been very successful for locating genes involved in Mendelian diseases or so called single gene disorders. However, the parametric linkage approach assumes the presence of a single disease locus that accounts for the majority of the genetic variance and enquires to specify the genetic model, including the mode of inheritance, disease allele frequency and penetrance of each genotype. Conversely, diseases like T2D have a complex mode of inheritance meaning that they are caused by multiple genetic variants interacting with each other and their environment and therefore the parametric linkage approach is not suitable.[83]

### *1.3.2 Non-parametric linkage analysis*

For complex disorders, non-parametric linkage methods have been developed. These methods are based on the principle that if a marker locus is closely linked to a locus underlying the disease or quantitative trait of interest, pairs of relatives that share alleles identical by descent (IBD) will have more-similar trait values than pairs of relatives that share no alleles IBD. Alleles that are shared IBD are always originated from a common ancestor (see Figure 6). The advantages of non-parametric linkage compared to parametric linkage are that no assumption about the mode of inheritance is required and small families can be used. These are easier to recruit.[82,84]

When inbreeding is absent, two unrelated people are expected to share 0 alleles IBD, a parent-offspring pair 1 allele IBD, MZ twins 2 alleles IBD and a sib-pair 0, 1 or 2 alleles IBD with a probability of 0.25, 0.5 and 0.25, respectively. If the parents in a nuclear family have unique alleles at a marker locus of interest, determining the exact IBD status for the offspring will be very straightforward (see Figure 6). However, the genotypes of the parents are not always fully informative and in case of late-onset disorders parental genotypes are often unavailable. In these situations, IBD allele sharing cannot be determined unequivocally and must be defined as a probability distribution where $\pi_0$, $\pi_1$ and $\pi_2$ denote the estimated probabilities of sharing 0, 1, or 2 alleles IBD, respectively. Once the three probabilities $\pi_0$, $\pi_1$ and $\pi_2$ of sharing 0, 1, or 2 alleles IBD have been estimated, the proportion of alleles shared IBD at the locus of interest is then calculated as $\hat{\pi} = \frac{1}{2}\pi_1 + \pi_2$.[85] For illustration, consider a nuclear family with the parents having genotypes AA and AB, and the offspring having the genotypes AB and AB. The probability distribution of alleles shared IBD for the offspring becomes $(\pi_0, \pi_1, \pi_2) = (0, \frac{1}{2}, \frac{1}{2})$ and the proportion of alleles shared IBD at the locus is $\frac{3}{4}$.[86]

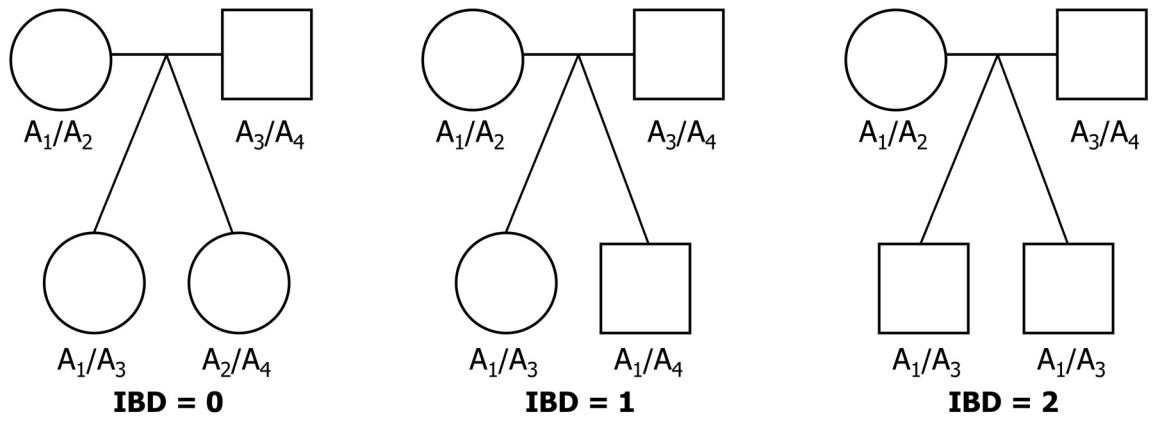


**Figure 6.** Examples of IBD allele sharing in dizygotic twin pairs.

In case parental genotypes are completely missing, IBD estimates can be constructed using allele frequencies.[85,87] However, in order to obtain informative IBD estimates it is of great importance to use highly polymorphic genetic markers. This is assessed by the heterozygosity of the marker, which corresponds with the likelihood that a randomly selected individual will be heterozygous for that marker. The heterozygosity (H) of a marker is dependent on the number of alleles ($A_1$, $A_2$, $A_3$ and etc) and their frequency ($f_1$, $f_2$, $f_3$ and etc), since $H=1-\sum f_i^2$. In addition, greater accuracy of the IBD estimation can be obtained by using the genotype information of multiple markers.[88,89]

*Sib-pair based approaches: qualitative traits*

Sib-pairs are extensively used to study the genetics of complex disorders and several non-parametric linkage methods have been developed. A commonly used non-parametric linkage method is the affected sib-pair (ASP) approach where large numbers of nuclear families with at least 2 affected children per family are analysed.[83,90] If a maker is not linked to the disease locus, the probabilities of sharing 0, 1 and 2 alleles IBD for sib-pairs from a non-inbred family are 0.25, 0.50 and 0.25, respectively. If the affected sib-pairs share more alleles IBD than expected by chance, the genetic marker is considered to be linked to the disease locus.[83,90] An alternative for the ASP approach is the discordant sib-pair (DSP) approach, which is based on the assumption that if a genetic marker is linked to the disease locus then discordant sib-pairs share less alleles IBD than would be expected by chance.[91] The DSP is thought to be more powerful than the ASP, since not only shared genetic factors but also shared environmental factors might contribute to phenotypic concordance in sib-pairs.[86]

*Sib-pair based approaches: quantitative traits*

A major disadvantage of both the ASP and DSP approach is that large numbers of affected and discordant sib-pairs are hard to obtain. Studying disease-related quantitative traits can circumvent this problem, because their variation can be studied in unaffected sib-pairs. Complex diseases like T2D and cardiovascular disease are very suitable for this type of analysis, since they are often a result of prolonged clustering of several metabolic abnormalities like obesity, glucose intolerance/insulin resistance, hypertension and/or dyslipidaemia. In addition, it is thought that studying intermediate traits is even a more powerful approach, since genes that influence traits at a lower level of a physiological hierarchy might be easier to map because the phenotypes they influence are closer to the "genetic substrate" that determines them. Hence, the total number of genes influencing these intermediate traits are likely to be fewer than the total number of genes involved in the disease.[92] Furthermore, the knowledge gained from this approach will increase the understanding of the molecular determinants of these metabolic pathways.
A commonly used sib-pair based linkage method to analyse whether a genetic marker is linked to a quantitative trait locus (QTL) is the Haseman-Elston regression method.[85] Haseman-Elston regression is based on the principle that if a marker is linked to a QTL, sibs sharing 2 alleles IBD are expected to have a smaller difference in trait values then sibs sharing 0 alleles IBD. The linear regression model ($Y = a + bX$) used, includes the squared difference in trait values between members of a sibling pair (Y) as the dependent variable

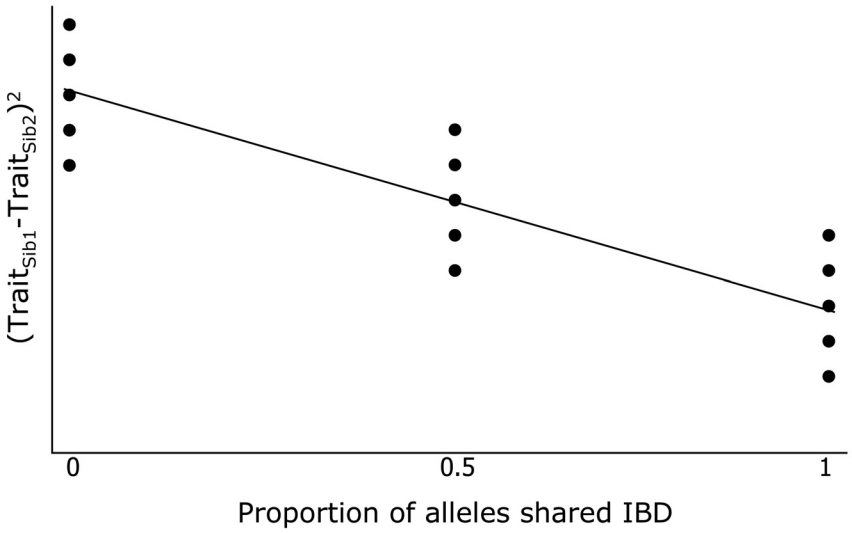


**Figure 7.** Haseman-Elston regression under linkage.

and the proportion of alleles shared IBD between them (X) at the marker locus as the independent variable. If there is no linkage between the genetic marker and a locus underlying the trait of interest, the regression coefficient will be equal to zero. However if there is linkage, sib-pairs that differ markedly for the phenotype of interest will share less alleles IBD than sib-pairs that show only small phenotypic differences. This will result in a regression coefficient smaller than zero (see also Figure 7).[85]

Variance component modelling can also be used to carry out linkage analysis, which is based on the principle that sib-pairs who share more alleles IBD at a QTL have higher correlated phenotypic values than sib pairs that share less alleles IBD. The traditional variance-covariance matrix is extended with an additional genetic component Q, which reflects the effect of the QTL. The correlation for the genetic component Q between sibs is equal to the proportion of alleles shared IBD at that locus. The expected variance–covariance matrices for MZ and DZ twins are defined as:

$$\textbf{AEQ}\quad MZ\ \begin{matrix} \\ T1 \\ T2 \end{matrix}\begin{matrix} \begin{matrix} T1 & T2 \end{matrix} \\ \begin{bmatrix} a^2 + e^2 + q^2 & a^2 + q^2 \\ a^2 + q^2 & a^2 + e^2 + q^2 \end{bmatrix} \end{matrix} \qquad DZ\ \begin{matrix} \\ T1 \\ T2 \end{matrix}\begin{matrix} \begin{matrix} T1 & T2 \end{matrix} \\ \begin{bmatrix} a^2 + e^2 + q^2 & \tfrac{1}{2}a^2 + \hat{\pi} q^2 \\ \tfrac{1}{2}a^2 + \hat{\pi} q^2 & a^2 + e^2 + q^2 \end{bmatrix} \end{matrix}$$

where $\hat{\pi} = \tfrac{1}{2}\pi_1 + \pi_2$, $\pi_1$ = probability of 1 allele shared IBD and $\pi_2$ = probability of 2 alleles shared IBD.[93] The effect of the QTL is evaluated by comparing the fit of the full model (including Q) with the model lacking Q (see also Figure 8).

DZ twins are preferred above normal sib-pairs, since they are matched for age and for a broad range of pre- and postnatal factors.[94] MZ twins are however uninformative for linkage and normally not included in variance components linkage analyses. However, variance components will be estimated more accurately when phenotypic data of MZ twins are included, which might result in greater power to detect a QTL.

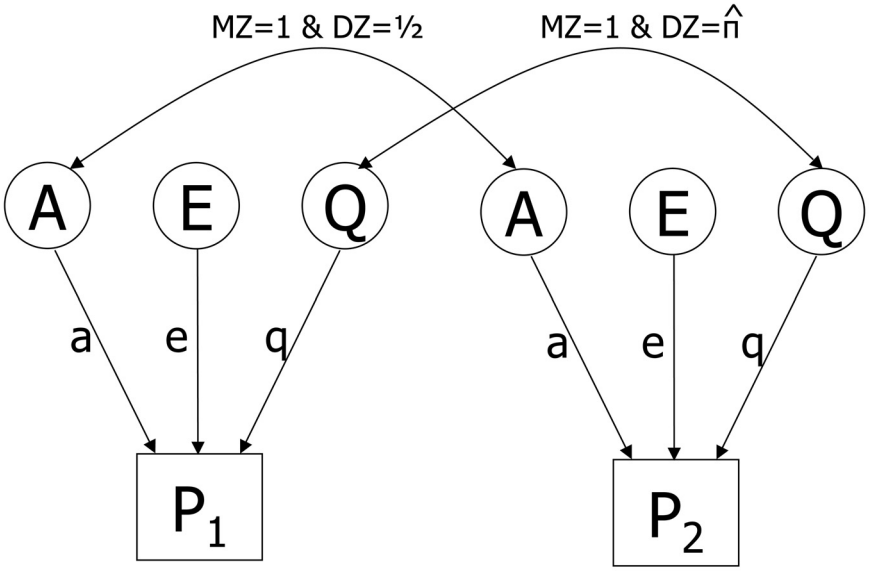


**Figure 8.** Path diagram for univariate variance components QTL linkage analysis. The additive genetic correlation is 1 for MZ and ½ for DZ twins. The correlation between the QTL is 1 for MZ twins and $\hat{\pi}$ (the estimated proportion of alleles shared IBD at the marker locus) for DZ twins.

## 1.4 ASSOCIATION STUDIES

Association studies are often carried out to follow up on linkage findings, which is the so called positional candidate gene approach, or can be of the functional candidate gene type, where genes are selected based on their potential involvement in the pathogenesis of the disease of interest. In addition, because of rapid developments in genotyping technology it has in the meantime become possible to perform association studies on a genome wide scale. However, the costs of this approach are still very high.
Linkage studies differ from association studies in perspective that linkage studies test for co-segregation of genetic markers and a disease phenotype within families, while association studies look at co-occurrence of a genetic marker and a disease at the level of the general population. Therefore association can be carried out using either related or unrelated individuals. In linkage studies different alleles can be correlated with the disease or trait of interest between families, because random recombination events change linkage relations across families. However when a marker is closely located to the causative genetic variant, it is unlikely that they are separated by recombination and are said to be in linkage disequilibrium (LD). Association with the causative genetic variant or a variant in LD with the causative variant will be the same both within and across families. Because linkage disequilibrium is sustained over much shorter distances than recombination, many more markers are needed for association studies compared to linkage studies, however association studies are more powerful to detect disease genes with small effects.[95,96]

### *1.4.1 Indirect and direct association studies*

Association studies can be subdivided into indirect and direct association studies. Indirect association studies intend to uncover functional polymorphisms via their non-random

association (linkage disequilibrium (LD)) with the genotyped single nucleotide polymorphisms (SNPs). It has been shown that most of the common SNPs in the genome are highly correlated with neighbouring SNPs and therefore the genotype of one SNP highly predicts the genotype of correlated adjacent SNPs. Thus instead of genotyping all SNPs in a candidate region of interest, indirect association studies target only a subset of SNPs (tagging SNPs) that capture the total sequence variation. The power of this approach is highly dependent on the efficiency of the selected tagging SNPs, but in general indirect association is less powerful then direct association.[96,97]
Direct association studies target putative functional polymorphisms in candidate genes of interest, like for instance non-synonymous SNPs leading to an amino acid change. In Caucasians, only 10.000–15.000 non-synonymous SNPs with a minor allele frequency >1% have been identified.[96] However, polymorphisms located in the promoter sequence might effect gene expression and polymorphisms located in the 5' and 3' untranslated region (UTR) might modify mRNA stability or translation and are also putative functional polymorphisms. If the targeted polymorphisms are truly functional, then this approach is the most powerful and is therefore applied in this thesis.[96]

### *1.4.2 Qualitative traits*

In the case-control design the allele and/or genotype frequencies of the variant of interest are compared between affected (cases) and unaffected (controls) individuals. A significantly higher allele or genotype frequency in the cases compared to the controls indicates that the variant of interest or a variant in LD with it might predispose to the disease.[96] The case-control design is the most commonly used study design for association studies, probably because unrelated individuals are required who are easier to recruit than family based-samples. However the selection criteria used to classify cases and controls are highly determinative for success of outcomes. For instance, controls have to be selected from the same population as the cases, since heterogeneity between cases and controls could result in spurious associations (population stratification).[83] Moreover, in diseases with a variable age of onset like T2D, the age of the control group is very important, since a too young control group might represent a mixture of subjects who will never develop the disease and those who will develop the disease later in life.
Problems related to selection of the control group, can be avoided by using a family-based association study. A very robust design makes use of nuclear families consisting of one or more affected offspring and at least one parent who is heterozygous for the SNP of interest, which are used to perform the transmission disequilibrium test (TDT). The TDT evaluates the frequency with which both alleles are transmitted to the affected offspring. If both alleles are equally transmitted to the offspring, then there is no association with the SNP and the disease. If one allele is more frequently transmitted than the other allele, the SNP is associated with the disease.[98] A major disadvantage of this approach is that nuclear families are harder to obtain then unrelated individuals and in case of late onset diseases, parental DNA is often not available.

### *1.4.3 Quantitative traits*

In addition to the case-control design and the use of nuclear families described above, association studies can also be performed using quantitative traits. Statistically significant

different mean values across the genotype groups, indicate that the studied polymorphism is involved in the trait of interest. Association analyses using quantitative traits can be performed using either unrelated and related individuals.[96] However, using related individuals complicates the statistical analysis, since standard linear regression assumes that the observations are independent.
Although MZ twins are uninformative for linkage, they are informative for association. In addition, using MZ twins for association reduces genotyping costs with 50%, because one genotype is associated with two phenotypes.

## 1.5 GENOMIC IMPRINTING

In general, both alleles are transcriptionally active. However, in some cases either the maternally or paternally inherited allele is exclusively expressed, while the other is silenced. This parent-of-origin dependent gene expression is called genomic imprinting and is primarily due to the epigenetic modification: DNA methylation. DNA methylation occurs when a methyl group is added to the nucleobase cytosine. When methyl groups are added to CpG-rich regions or so called CpG islands, hypermethylation occurs. Since CpG islands are predominantly located in promoter regions, hypermethylation often results in transcriptional silencing of a gene. On the other hand, loss of DNA methylation or hypomethylation in a promoter region will result in transcriptional activation.[99]
One of the candidate genes studied in this thesis is maternally imprinted (i.e. insulin-like growth factor 2 (*IGF2*)). Since in traditional linkage analysis the paternal and maternal inherited alleles are equally weighted, genomic imprinting decreases the power of the traditional linkage analysis. To asses the parent-of-origin effect, the variance due to the QTL needs to be partitioned into a component that reflects the QTL effect of the maternally derived chromosome ($Q_M$) and a component reflecting the QTL effect of the paternally derived chromosome ($Q_P$). The correlation for the genetic component $Q_M$ between sibs is equal to the proportion of maternal alleles shared IBD at that locus, and the correlation for the genetic component $Q_P$ is equal to the proportion of paternal alleles shared IBD at that locus. The expected variance–covariance matrices for MZ and DZ twins are defined as:

$$\mathbf{AEQ_MQ_P} \quad \text{MZ} \quad \begin{array}{c} \\ \text{T1} \\ \text{T2} \end{array} \begin{array}{c} \begin{array}{cc} \text{T1} & \text{T2} \end{array} \\ \begin{bmatrix} a^2 + e^2 + q_M^2 + q_P^2 & a^2 + q_M^2 + q_P^2 \\ a^2 + q_M^2 + q_P^2 & a^2 + e^2 + q_M^2 + q_P^2 \end{bmatrix} \end{array}$$

$$\mathbf{AEQ_MQ_P} \quad \text{DZ} \quad \begin{array}{c} \\ \text{T1} \\ \text{T2} \end{array} \begin{array}{c} \begin{array}{cc} \text{T1} & \text{T2} \end{array} \\ \begin{bmatrix} a^2 + e^2 + q_M^2 + q_P^2 & \tfrac{1}{2}a^2 + \pi_M q_M^2 + \pi_P q_P^2 \\ \tfrac{1}{2}a^2 + \pi_M q_M^2 + \pi_P q_P^2 & a^2 + e^2 + q_M^2 + q_P^2 \end{bmatrix} \end{array}$$

$\pi_M$ is calculated by summing the probability that both maternal and paternal alleles are shared IBD plus the probability that only the maternal allele is shared IBD. $\pi_P$ is calculated by summing the probability that both maternal and paternal alleles are shared IBD plus the probability that only the paternal allele is shared IBD. $\pi_M$ and $\pi_P$ can be either 0.0 and 0.5, because $\pi_M + \pi_P = \hat{\pi}$.

Genomic imprinting has also implications for association studies. When parental DNA is unavailable only homozygote subjects can be included in the statistical analyses, resulting in a considerable loss of power.

## 1.6 CANDIDATE GENE APPROACH

In general, linkage is used to identify candidate loci on a genome wide scale. To uncover genes involved in T2D, numerous genome-wide linkage scans have been carried out and evidence for linkage at several chromosomal regions has been reported.[100-106] Usually, these regions are large and contain many genes and therefore candidate genes in subsequent studies are often selected based on pre-existing knowledge. Instead of genotyping markers across the whole genome, in this thesis linkage was assessed directly by selecting polymorphic microsatellite markers located within or near functional candidate genes (encoding proteins with a suspected role in the pathophysiology of T2D). Linkage to metabolic traits in these candidate gene regions provides evidence for the possible presence of genetic variation in these regions influencing these traits. In addition to linkage, we also carried out association analyses with SNPs genotyped in functional candidate genes.
The candidate genes studied in this thesis include:

- ATP-binding cassette, sub-family C, member 8 (*ABCC8*): *ABCC8* encodes the regulatory β subunit sulfonylurea receptor (SUR1) of the ATP-sensitive potassium ($K_{ATP}$) channel in pancreatic β-cells and neurons. Closure of potassium channels in pancreatic β-cells triggers insulin release.[107]
- Adiponectin (*ADIPOQ*): Adiponectin is an adipocyte-derived hormone that acts through binding to the adiponectin receptor 1 and 2 (*ADIPOR1* and *ADIPOR2*). Serum adiponectin levels are inversely correlated with obesity, insulin resistance and hyperlipidemia. Experiments in rodents have demonstrated that adiponectin has a strong insulin-sensitising effect.[108]
- Angiotensin II type I receptor (*AGTR1*)**:** AGTR1, part of the renin-angiotensin system, is activated by angiotensin II, which stimulates vasoconstriction, and regulates cellular growth and body fluid homeostasis.[109] Anti-hypertensive treatments that block the renin-angiotensin system by antagonising AGTR1 cause an improved glucose homeostasis.[110,111]
- Cytochrome 1A1 (*CYP1A1*): *CYP1A1* encodes a cytochrome P450 enzyme that is involved in both metabolic activation and detoxification of polycyclic aromatic hydrocarbons (present in cigarette smoke).[112,113] Although, *CYP1A1* has mainly been studied in relation to lung cancer, recently a common polymorphism in *CYP1A1* has been associated with low weight at birth.[114]
- Ectonucleotide pyrophosphatase/phosphodiesterase 1 (*ENPP1*): ENPP1, also known as plasma cell membrane glycoprotein-1 (PC1), inhibits insulin receptor signalling by interacting with the extracellular α-subunit, thereby inhibiting autophosphorylation of the insulin receptor and subsequently downstream signalling.[115,116]
- Fatty acid amide hydrolase (*FAAH*): FAAH degrades endogenous cannabinoids that stimulate food intake through binding to the cannabinoid type 1 (CB1) receptor in the hypothalamus. In addition, experiments in rodents suggest that the endogenous

cannabinoid system has also direct peripheral metabolic effects, including modulation of adipocyte energy metabolism and stimulating hepatic lipogenesis.[117]

- Glutamate decarboxylase 2 (*GAD2*): GAD2, expressed in the brain and pancreatic islets, encodes the glutamic acid decarboxylase enzyme (GAD65) that catalyses the formation of the neurotransmitter γ-aminobutyric acid (GABA). In the hypothalamus, GABA stimulates food intake possibly by interacting with neuropeptide Y.[118,119] Mice overexpressing *GAD2* showed increase GABA release from pancreatic ß-cells, and exhibited impaired glucose tolerance and inhibition of the glucose-induced insulin release, which is possibly the result of reduced glutamate availability.[120,121]
- Glucokinase (*GCK*): Glucokinase (hexokinase 4), mainly expressed in pancreatic β-cells and hepatocytes, phosphorylates glucose into glucose-6-phosphate.[122]
- Insulin-like growth factor 1 (*IGF1*): IGF1 is besides insulin the second most powerful peptide with glucose lowering effects. IGF1 exerts its actions by binding to the IGF1 receptor (IGF1R) and, although with lower affinity, to the insulin receptor. IGF1 stimulates growth and promotes peripheral glucose uptake, diminishes hepatic glucose production and improves the blood lipid profile.[123]
- Insulin-like growth factor 2 (*IGF2*): The *IGF2* gene is maternally imprinted and paternally expressed. IGF2 acts through binding to the IGF1R, and binds together with insulin (not IGF1) to the insulin receptor INSR-A isoform, which is preferentially expressed during fetal growth, but IGF2 can also bind to the classical INSR-B isoform.[124,125] In addition, IGF2 has also high affinity for the IGF2 receptor (IGF2R), that mediates IGF2 degradation.[125] *IGF2* plays a key role during fetal development. After birth *IGF2* is transcribed at a lower level, but several studies suggest that IGF2 has postnatally some metabolic effects as well.[126-128]
- Insulin-like growth factor binding protein 1 (*IGFBP1*): IGFBP1 is a member of the IGFBP family that prevents the insulin-like activity and regulate the bioavailability of IGF1 and IGF2.[129] Insulin inhibits hepatic IGFBP1 production and plasma IGFBP1 levels are positively correlated with insulin sensitivity and inversely correlated with fasting plasma insulin levels.[130]
- Interleukin-6 (*IL6*): IL6 is a pro-inflammatory cytokine which is secreted by a large number of cell-types, including immune cells, muscle cells, endothelial cells, hepatocytes, pancreatic ß-cells and adipocytes.[131] Plasma interleukin-6 levels are positively correlated with fasting insulin and obesity, and predict future development of T2D.[132,133] Currently, there is no consensus on whether IL6 affects insulin release in pancreatic ß-cells or contributes to insulin resistance in muscle and adipose tissue, but there is quite some evidence demonstrating that IL6 might inhibit insulin signalling in hepatocytes.[131]
- Insulin receptor (*INSR*): Binding of insulin and with lower affinity IGF1 and IGF2 to the insulin receptor initiates phosphorylation of intracellular substrates leading to the activation of downstream signalling pathways that mediate metabolic and growth-promoting effects.[20,123]
- Insulin receptor substrate (*IRS2*): IRS2 belongs to the family of insulin receptor substrate (IRS) proteins that are directly phosphorylated by the INSR or the IGF1R, resulting in the activation of downstream signalling proteins.[20] IRS2 mediates the effect of insulin on hepatic glucose production, gluconeogenesis and glycogen synthesis.[43]

- Potassium inwardly-rectifying channel, subfamily J, member 11 (*KCNJ11*): *KCNJ11* encodes the pore-forming, inwardly rectifying potassium channel α-subunit (Kir6.2) of the ATP-sensitive potassium ($K_{ATP}$) channel in many tissues, including pancreatic β-cells, brain, heart and skeletal muscle. Closure of potassium channels in pancreatic β-cells triggers insulin release.[107]
- Leptin (*LEP*) and the leptin receptor (*LEPR*): Leptin, which carries out its biological activities through binding to the leptin receptor, is primarily secreted in adipose tissue and leptin plasma levels correlate positively with fat mass.[37] Leptin inhibits food intake and increases energy expenditure by stimulating the anorexigenic pro-opiomelanocortin (POMC)/cocaine- and amphetamine-regulated (CART) neurons, and inhibiting the orexigenic neuropeptide Y (NPY)/agouti-related protein (AGRP) neurons in the arcuate nucleus of the hypothalamus. In addition, it has been demonstrated that leptin controls hepatic glucose production, promotes lipolysis and inhibits lipogenesis in adipose tissue.[134-137]
- Peroxisome proliferator activated receptor-γ (*PPARγ*): PPARγ, highly expressed in adipose tissue, is a key transcription factor in the differentiation of preadipocytes into adipocytes, and improves NEFA uptake and triglyceride storage in adipose tissue. Accordingly, stimulation of PPARγ activity lowers NEFA plasma levels and improves insulin sensitivity.[32] In addition, it has been suggested that PPARγ mediates glucose transport and glycolysis in the pancreatic ß-cells and liver by stimulating the expression of GLUT2 and glucokinase.[138]
- Peptide YY (*PYY*): PYY is postprandially secreted from endocrine L-cells of the intestine in proportion to the meal calorie content. PYY suppresses appetite and inhibits food intake by binding to the neuropeptide Y receptor Y2 (NPY2R) that has an inhibitory effect on the orexigenic NPY neurons of the arcuate nucleus in the hypothalamus. In addition, fasting PYY levels are negatively correlated with obesity.[139,140]
- Resistin (*RETN*): In mice, resistin is mainly produced by adipocytes and over-expression of resistin induces hepatic insulin resistance and dyslipidaemia.[141-143] In humans, where macrophages are the major source of resistin in adipose tissue,[144] the role of resistin is less clear. Serum resistin levels correlate poorly with waist-to-hip ratio, triglycerides and insulin resistance.[145,146] However, in T2D patients it has been demonstrated that resistin levels are correlated with hepatic insulin resistance.[147] In addition, resistin levels have been associated with inflammatory markers, suggesting a role in inflammatory processes.[148,149]
- Uncoupling protein 2 (*UCP2*): UCP2 is a member of the uncoupling protein family that are located on the inner membrane of the mitochondria. UCPs mediate a mitochondrial proton leak, thereby uncoupling substrate oxidation from ATP synthesis. Thus generating heat instead of ATP. Increased UCP2 expression resulting in an increased waste of energy might protect against obesity. However, increased UCP2 expression in ß-cells results in a decreased insulin secretion, possibly contributing to an increased T2D risk. In addition, enhanced UCP2 expression might inhibit reactive oxygen species production, thus possibly protecting against atherosclerosis.[150]

## 1.7 AIMS OF THIS THESIS

In this thesis we aim to identify genetic variants underlying quantitative traits that predispose to or are associated with T2D, measured in a young twin sample recruited from the East Flanders Prospective Twin Study (EFPTS). The EFPTS is a population-based twin register, which has been recording all multiple births born in the Belgium province of East Flanders since 1964.[73] Between 1964 and 2005, 6973 twin pairs were registered. Compared to other twin registries, the EFPTS has several unique features:

- The register is population based and prospective.
- The twins are ascertained at birth.
- Several basic perinatal data are collected at birth, including birth weight, length and head circumference, ponderal index and gestational age.
- Placental morphology is recorded, including the number of placenta, weight, size, cord insertion and vascular anastomosos.
- Foetal membranes, i.e. number of amnion and chorion membranes, are examined and zygosity is determined.
- Since 1969 placental biopsies have been taken and stored at –20°C.

Worldwide, the EFPTS is the only large twin registry that can discriminate between the subtypes of MZ twins.[73] In this thesis we use a subset of young adults from the EFPTS, comprising 380 complete twin pairs and 44 single twins, of which phenotypic measurements have previously been determined, including birth weight, parameters related to obesity, the carbohydrate and lipid metabolism.[78]

The aims of this thesis are:

- To estimate the heritabilities of a series of metabolic risk factors that predispose to or are associated with T2D in the studied twin sample. In addition, compare MZ MC and MZ DC intra-pair correlations to determine whether chorion type biases the heritability estimates of the studied traits (Chapter 2).
- To determine whether polymorphic microsatellite markers located close to the *ABCC8*, *ADIPOQ*, *GCK*, *IGF1*, *IGFBP1*, *INSR*, *LEP*, *LEPR*, *PPARγ* and the *RETN* genes are linked to the T2D related metabolic risk factors of interest and evaluate whether incorporating phenotypic data of the MZ twin pairs improves univariate variance component linkage analysis (Chapter 3).
- To evaluate whether SNPs located in *AGTR1*, *CYP1A1*, *ENPP1*, *FAAH*, *GAD2*, *IL6*, *IRS2*, *KCNJ11*, *LEP*, *LEPR*, *PPARγ*, *PYY* and *UCP2* are associated with the T2D related metabolic risk factors by using a direct association approach where putative functional polymorphisms are targeted, including non-synonymous SNPs, SNPs located in the promoter sequence and SNPs located in the 5' and 3' UTR (Chapters 4 and 5).
- To evaluate whether genetic variation in the maternally imprinted *IGF2* gene explains part of the variation of T2D related metabolic risk factors by carrying out parent-of-origin specific linkage and association analyses using 3 SNPs and a microsatellite marker located in or in the close vicinity of the *IGF2* gene (Chapter 6).

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# CHAPTER 2

# ANTHROPOMETRY, CARBOHYDRATE AND LIPID METABOLISM IN THE EAST FLANDERS PROSPECTIVE TWIN SURVEY: HERITABILITIES

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## 2.1 ABSTRACT

We determined the genetic contribution to 18 anthropometric and metabolic risk factors of T2D using a young healthy twin population. Traits were measured in 240 monozygotic (MZ) and 138 dizygotic (DZ) twin pairs aged 18 to 34 years. Twins were recruited from the Belgian population-based East Flanders Prospective Twin Survey, which is characterised by its accurate zygosity determination and extensive collection of perinatal and placental data, including information on chorionicity. Heritability was estimated using structural equation modelling implemented in the Mx software package. Intra-pair correlations of the anthropometric and metabolic characteristics did not differ between MZ monochorionic and MZ dichorionic pairs; consequently heritabilities were estimated using the classical twin approach. For body mass, BMI and fat mass, quantitative sex differences were observed; genetic variance explained 84, 85 and 81% of the total variation in men and 74, 75 and 70% in women, respectively. Heritability estimates of the waist-to-hip ratio, sum of four skinfold thicknesses and lean body mass were 70, 74 and 81%, respectively. The heritability estimates of fasting glucose, fasting insulin, homeostasis model assessment of insulin resistance and beta cell function, as well as insulin-like growth factor binding protein-1 levels were 67, 49, 48, 62 and 47%, in that order. Finally, for total cholesterol, LDL-cholesterol, HDL-cholesterol, total/HDL-cholesterol ratio, triglycerides, non-esterified fatty acids (NEFA) and leptin levels, genetic factors explained 75, 78, 76, 79, 58, 37 and 53% of the total variation, respectively. Genetic factors explain the greater part of the variation in traits related to obesity, glucose intolerance/insulin resistance and dyslipidaemia.

## 2.2 INTRODUCTION

T2D is a heterogeneous disease that involves both genetic and environmental factors. Its incidence is rising rapidly worldwide and consequently much research is focused on the genetic components of the disorder, in order to get a better understanding of the pathogenesis and eventually to achieve better, more personalised diagnostics, treatment and prevention.[1]

The starting point in the search for genes is to estimate the degree of heritability of intermediate traits leading to the disease in the studied population. Heritability is the proportion of phenotypic variation of a trait that can be attributed to genetic variation.[2] The degree of heritability is an important determinant of the power to detect and localise disease-related genes.[3] Although heritabilities can in principle be estimated from all kinds of related individuals, twin studies allow the variation to be split up into genetic, shared environmental and unique environmental components, thus offering one of the most valid estimations.[2]

A variety of studies estimating heritabilities of risk factors of T2D in adult twins have been carried out. A summary of the larger studies (>200 twin pairs) on the risk factors obesity, glucose intolerance/insulin resistance and dyslipidaemia is provided in Table S1. The heritability of BMI, the most frequently used measure of obesity, has been estimated extensively and ranges between 40 and 90%.[4-21] For all the other traits, fewer studies have been performed; the total variation of fasting glucose and fasting insulin is explained by genetic factors for 12 to 50%[22,23] and 14 to 54%,[7,18,22-24] respectively; for total cholesterol,

LDL-cholesterol, HDL-cholesterol and triglycerides heritability estimates range between 0 and 98%.[7,10,13,17,21,25-34] In addition to sample numbers and statistical methodologies used, the large variation in heritability estimates may also be caused by the genetic background of the populations studied and the environmental exposures experienced (see Table S1). For the majority of intermediate traits related to T2D, the number of larger studies performed is quite small and studies in which heritabilities of several risk factors of T2D were estimated in the same population are even scarcer (Table S1).
To determine the genetic contribution to T2D, we used variance components modelling to estimate the heritabilities of 18 anthropometric and metabolic risk factors of the disease, including parameters quantifying obesity, glucose intolerance/insulin resistance and dyslipidaemia. In addition, we also determined the heritabilities of the hormones insulin-like growth factor binding protein 1 (IGFBP1), which has been shown to be related to several cardiovascular risk factors, and leptin, which plays a role as satiety signal regulating body composition and energy expenditure.[35] The characteristics were measured in 756 healthy twins, divided into 240 monozygotic (MZ) and 138 dizygotic (DZ) twin pairs in the age range of 18 to 34 years, recruited from the East Flanders Prospective Twin Survey (EFPTS).

## 2.3 MATERIALS AND METHODS

### *2.3.1 Participants*

The EFPTS is a population-based twin register, which started in 1964 and has been recording all multiple births in the Belgian Province of East Flanders until the present day. A detailed description of the EFPTS has been published.[36] Zygosity was determined using sequential analysis based on sex, fetal membranes, umbilical cord blood groups, placental alkaline phosphatase and DNA marker analysis. Between July 1964 and May 1982, the Twin Survey had registered 2,141 twin pairs who met the World Health Organisation criteria for live born infants (birth weight ≥ 500 g or gestational age ≥ 22 weeks, if birth weight unknown). Pairs of whom one or both members were stillborn, died in neonatal or later life or suffered from major congenital malformations were excluded. We randomly contacted 803 pairs using an envelope system. To assure equally distributed groups, we stratified for birth year and zygosity. Since the twin survey was originally set up to determine the effect of chorionicity, the number of MZ twin pairs was over sampled. Eventually, 424 twin pairs (52.7%) volunteered to participate in the study. Sex, gestational age and birth weight did not differ between participants and non-participants (i.e. those who where eligible, but refused to participate or had not been contacted) ($p$>0.05), although participants were slightly older than the non-participants and the proportion of monochorionic (MC) twins was higher in the group of participants ($p$<0.05). For the present analysis participants suffering from type 1 diabetes were excluded and only twin pairs of whom both members participated were incorporated. Consequently, phenotypic data were available for 378 complete pairs, consisting of 113 monozygotic men (MZM), 127 monozygotic women (MZF), 46 DZ men (DZM), 49 DZ women (DZF) and 43 DZ opposite sex (DZOS) pairs. Additionally, for the analysis of lipid and carbohydrate parameters, participants taking drugs with potential effects on lipid or carbohydrate metabolism were excluded (n=11). Twins were randomly assigned to be the first or the second member of a pair. The Ethics Committee of the Faculty of

Medicine of the Katholieke Universiteit Leuven approved the project and all participants gave informed consent.

### 2.3.2 Phenotypic measurements

Between February 1997 and April 2000, the twins visited the research centre in Leuven for a 2 h examination, which started in the morning after an overnight fast. Participants were measured barefoot and lightly clothed. Standing height (cm) was measured to the nearest 0.1 cm with a Harpenden fixed stadiometer (Holtain, Crosswell, UK). Body mass (kg) was measured on a balance scale (SECA, Hamburg, Germany) to the nearest 0.1 kg. Body mass index (BMI) was calculated as body mass divided by the square of height ($kg/m^2$). Waist and hip circumferences were measured with a flexible steel tape to 1 mm accuracy. Waist circumferences were taken at the smallest point between the costal margin and the iliac crest and hip circumference at the widest part of the hips, generally at the level of the greater trochanters. Waist-to-hip circumference ratio (WHR) was expressed as a percentage. Lean body mass was measured using a bioelectrical impedance analyser (BIA310; Biodynamics, Seattle, WA, USA). Fat mass (kg) was calculated by subtracting the value for lean body mass from total body mass. Four skinfold thicknesses were taken in duplicate, to 0.1 mm accuracy with a Harpenden skinfold calliper (British Indicators, St Albans, UK) at the biceps, triceps, subscapular and suprailiac. The four thicknesses were summed (S4SF) to evaluate the overall subcutaneous fatness. Blood samples were taken to measure plasma hormone concentrations. Plasma leptin was measured with an immunoradiometric assay in a coated tube (Diagnostic Systems Laboratories, Webster, TX, USA). Plasma lipids (triglycerides, total cholesterol and HDL-cholesterol) were measured on an auto-analyser (AU600; Olympus, Kyoto, Japan). LDL-cholesterol was estimated using Friedewald's formula.[37] Non-esterified fatty acids (NEFA) were measured using a colorimetric assay with the optical density measured at 550 nmol/l. Plasma glucose was measured using the hexokinase method (Olympus AU600). Plasma insulin was determined using a microparticle enzyme immunoassay (Axsym; Abbott Laboratories, Chicago, IL, USA). Insulin-like growth factor binding protein 1 (IGFBP1) was measured by radio-immunoassay, as described.[38] Homeostasis model assessment (HOMA) was used to assess insulin resistance and beta cell function.[39]

### 2.3.3 Descriptive statistical analysis

Anthropometric and metabolic characteristics are expressed as mean ± SD according to chorion type, for men and women separately. BMI, sum of four skinfold thicknesses (S4SF), fat mass, IGFBP1, fasting insulin, insulin resistance, beta cell function, leptin, total/HDL-cholesterol ratio and triglycerides values had a skewed distribution. After transforming these data to a natural logarithmic scale, a normal distribution was obtained and the transformed data were used when performing statistical tests. Differences in means between MZ MC, MZ dichorionic (DC) and DZ twins were calculated using the PROC MIXED method implemented in SAS version 9.1 (SAS Institute, Cary, NC, USA). A random intercept model was used, where the intercept of each twin pair was modelled as a function of the population intercept plus a unique contribution of the twin pair. In addition, we allowed the variance–covariance structure of the random intercept to differ between MZ MC, MZ DC and DZ pairs. Differences in means were considered significant if the 2 *df* F test indicated $p<0.05$.

For all traits, effects of potential covariates were also checked using the random intercept model of PROC MIXED, where the variance–covariance structure of the random intercept was allowed to differ between MZ and DZ pairs. The variables BMI, WHR and S4SF were checked for the effect of potential confounding by sex and age; body mass, fat mass and lean body mass were checked for the effect of sex, age and height; the blood parameters were checked for the effect of sex, age and fat (BMI, WHR or S4SF). Covariates were considered significant if $p<0.05$. Intra-pair correlation coefficients were calculated for MZ MC and MZ DC twin pairs and for each of the five sex by zygosity groups (MZM, MZF, DZM, DZF and DZOS) before and after adjustment for significant covariates using the Mx software package.[40] In addition, we used a linear regression analysis to test whether twin correlations differed between MZ MC and MZ DC twin pairs before and after adjusting for covariates.

### *2.3.4 Twin model fitting*

Twin methodology makes use of the fact that MZ twins are genetically identical, whereas DZ twins share 50% of their segregating genes. Assuming that MZ and DZ twins share their common environment to the same extent, a higher concordance rate in MZ twins than in DZ twins reflects genetic influences. To estimate the genetic and environmental components of variance of the traits, twin model fitting of raw data was implemented using the statistical package Mx.[40] Scripts were downloaded from the GenomEUtwin Mx-script library (http://www.psy.vu.nl/mxbib/). Univariate twin analyses were performed, where the phenotypic variance can be decomposed into additive genetic (A, additive effects of genes on multiple loci), non-additive genetic (D, interactions between alleles at the same locus (dominance) or on different loci (epistasis)), common environmental (C, environmental effects shared by twins reared in the same family) and unique environmental effects (E, environmental effects unique to the individual). MZ twins are assumed to share the same A and D genetic variance; DZ pairs are assumed to share half of the A variance and a quarter of the D variance. The C variance is assumed to be the same for both MZ and DZ twin pairs. The broad sense heritability ($H^2$), which estimates the extent to which variation of a trait in a population can be explained by genetic variation, is defined as the proportion of genetic variance to total phenotypic variance. As non-additive genetic (D) and shared environmental effects (C) cannot be identified simultaneously in data from twins reared together, ACE and ADE models were fitted separately. The significance of variance components A, C or D in the model was tested by dropping these parameters and comparing the fit of the models.[5]
To test whether genetic and environmental factors influence a trait to the same degree in men and women, we compared a quantitative heterogeneity model (variance components free to differ across sexes) with a homogeneity model (variance components equal for both sexes). In addition, we verified whether the distribution of a trait differed among men and women by testing a scalar model, which assumes that the female variance components are common multiples of the male variance components. In this model the variance components were constrained to be equal for both sexes, but total variances were allowed to differ between men and women (see Figure 1).[5]

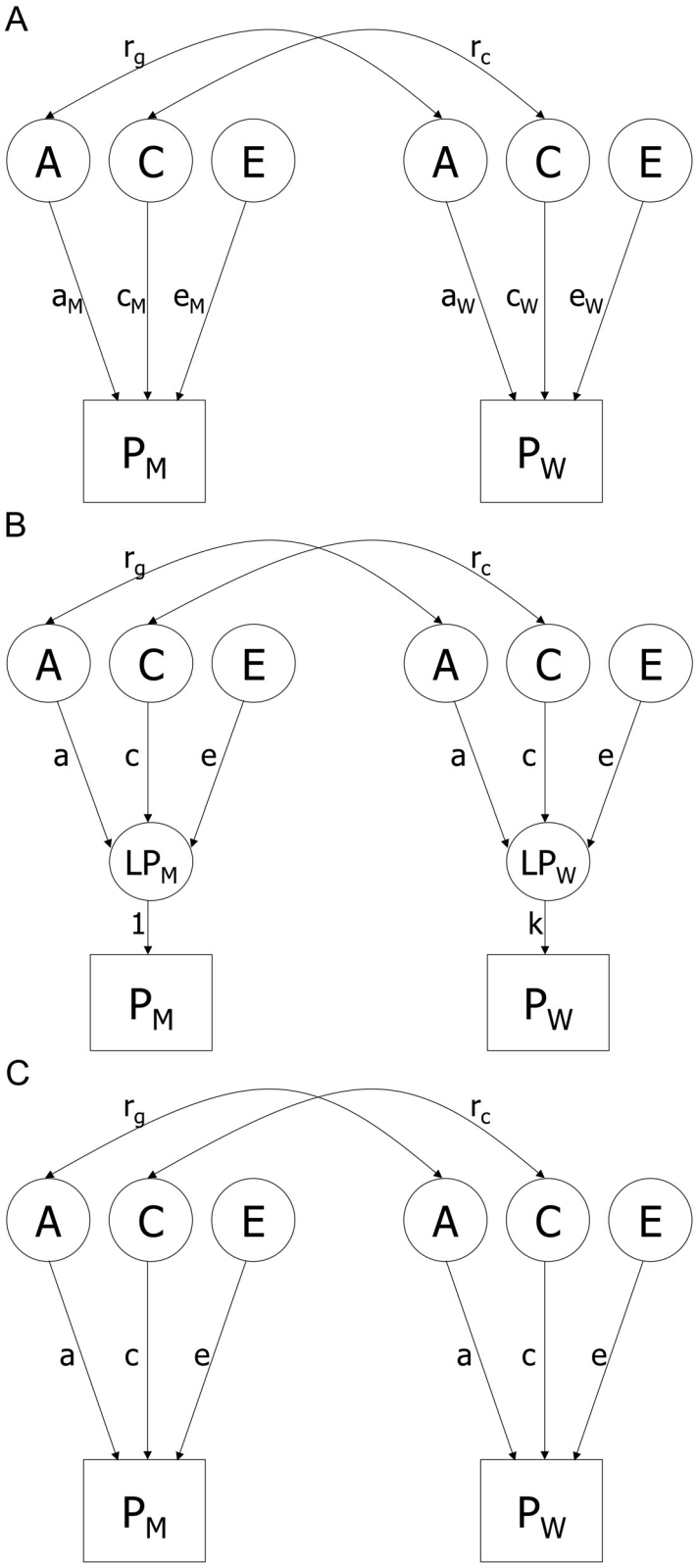


**Figure 1.** Path diagram for a univariate quantitative heterogeneity (**A**), scalar (**B**) and homogeneity model (**C**) only presenting opposite sex pairs. Observed phenotypes ($P_M$ and $P_W$) for the male and female twins are shown in rectangles, while latent factors (A, C and E) and latent phenotypes ($LP_M$ and $LP_W$) are shown in circles. Path coefficients of observed variables on the different latent factors are shown in lower case: a, additive genetic effect; c, common environmental effect; e, unique environmental effect; k, scalar factor. The genetic correlation is represented by $r_g$ (1 for MZ and 0.5 for DZ twins) and the common environmental correlation is represented by $r_c$ (1 for MZ and DZ twins).

If for a certain trait the correlation of the opposite-sex pairs is smaller than the correlations for the like-sexed DZ pairs, it is suggested that the correlation between additive genetic factors in opposite-sex pairs is smaller than 0.5. This indicates that different genetic factors influence this trait in men and women. To test this, a full heterogeneity model (variance components free to differ across sexes, plus the correlation between the additive genetic factors in opposite-sex pairs free to be estimated between 0 and 0.5) was compared with a quantitative heterogeneity model.
All variance components were estimated both unadjusted and adjusted for the covariates. In the models, phenotypic means were adjusted for significant covariates by modelling them as definition variables in the means model. The difference in fit between the nested models was evaluated with the likelihood ratio $\chi^2$ test, which uses the difference between the –2log likelihood of the full and the restricted model. This difference is distributed as $\chi^2$. The *df* of the test were calculated as the difference in *df* between the models. When the $\chi^2$ was not statistically significant ($p$<0.05), the most parsimonious model was selected, i.e. the model with the best fit given the number of *df*. When comparing the fit of non-nested models (e.g. ACE with ADE), the model with the lowest Akaike's information criterion (AIC) was preferred.

## 2.4 RESULTS

### *2.4.1 Descriptive statistical analysis*

The mean values of the anthropometric and metabolic characteristics of the twins are presented in Table 1 according to chorion type for men and women separately. In men, fasting glucose levels were lower in DZ twins than in MZ MC and MZ DC twins. In women, MZ DC twins were younger and had lower total cholesterol and LDL-cholesterol levels than MZ MC and DZ twins (Table 1).
The covariates adjusted for, the intra-pair correlations of MZ MC and MZ DC pairs, and the intra-pair correlations of each sex by zygosity group before and after adjustment are summarised in Table 2. Intra-pair correlations of the anthropometric and metabolic characteristics did not differ between MZ MC and MZ DC pairs ($p$>0.05). Adjusting for covariates strongly reduced the correlations of the leptin concentrations. The correlations of the other traits were only minimally affected by adjustment. Twin correlations for total cholesterol and LDL-cholesterol were high in both MZ and DZ twin pairs, indicating a common environmental effect. The DZ correlations of IGFBP1, fasting insulin and insulin resistance were less then half of the MZ correlations, suggesting that non-additive genetic effects might be important. Correlations of the other traits were in agreement with a model containing additive genetic and unique environmental influences (Table 2).

### *2.4.2 Twin model fitting*

The variance components and 95% confidence intervals (CIs) of the best fitting models before and after adjustment for covariates are presented in Table 3. The best fitting model for lean body mass and the obesity parameters body mass, BMI, WHR, S4SF and fat mass was an AE model containing a major genetic component. For total cholesterol and LDL-cholesterol, the ACE model was the best fitting model. However, after adjusting for

**Table 1.** Anthropometric and metabolic characteristics of the twin population according to chorion type for men and women separately in the EFPTS.

| | Men | | | | Women | | | |
|---|---|---|---|---|---|---|---|---|
| Characteristic | MZ MC | MZ DC | DZ | *p* | MZ MC | MZ DC | DZ | *p* |
| n | 134 | 92 | 135 | | 142 | 112 | 141 | |
| Age (years)[a] | 25.3±4.5 | 25.0±4.9 | 25.7±4.7 | 0.33 | 25.9±4.4 | 24.0±4.8 | 25.7±4.6 | 0.02 |
| Body height (cm) | 178.0±5.7 | 178.3±7.2 | 178.4±6.5 | 0.92 | 165.0±6.3 | 165.6±6.1 | 166.2±6.4 | 0.59 |
| Body mass (kg) | 70.7±10.5 | 69.6±8.4 | 70.9±10.2 | 0.67 | 60.5±9.7 | 60.5±10.6 | 60.8±10.2 | 0.98 |
| BMI ($kg/m^2$)[b] | 22.1±1.1 | 21.8±1.1 | 22.1±1.2 | 0.73 | 22.0±1.2 | 21.8±1.2 | 21.8±1.2 | 0.86 |
| WHR (%) | 83.4±5.4 | 83.4±5.8 | 82.8±5.5 | 0.84 | 73.4±4.5 | 73.3±4.6 | 72.5±4.3 | 0.30 |
| S4SF (mm)[b] | 35.9±1.5 | 34.6±1.5 | 34.8±1.5 | 0.89 | 57.1±1.4 | 55.6±1.5 | 53.6±1.4 | 0.50 |
| Fat mass (kg)[b] | 11.9±1.6 | 11.2±1.5 | 12.2±1.5 | 0.33 | 16.9±1.3 | 16.7±1.4 | 16.8±1.3 | 0.95 |
| Lean body mass (kg) | 57.7±6.9 | 57.6±6.0 | 57.7±6.6 | 0.99 | 42.9±5.1 | 43.1±5.6 | 43.4±6.0 | 0.89 |
| IGFBP1 (ng/ml)[b] | 11.0±1.9 | 10.3±1.8 | 11.5±1.9 | 0.46 | 16.8±2.0 | 16.6±2.2 | 18.4±2.3 | 0.58 |
| Fasting insulin (pmol/l)[b] | 33.5±1.5 | 32.7±1.6 | 32.7±1.6 | 0.94 | 38.6±1.5 | 35.8±1.6 | 40.1±1.5 | 0.24 |
| Insulin resistance (HOMA)[b] | 1.2±1.6 | 1.2±1.6 | 1.2±1.6 | 0.69 | 1.3±1.5 | 1.2±1.7 | 1.4±1.5 | 0.26 |
| Fasting glucose (mmol/l) | 5.0±0.5 | 4.9±0.4 | 4.8±0.4 | 0.01 | 4.5±0.3 | 4.6±0.4 | 4.6±0.4 | 0.23 |
| Beta cell function (HOMA)[b] | 77.2±1.5 | 78.2±1.6 | 86.5±1.7 | 0.10 | 135.2±1.7 | 115.7±1.6 | 126.7±1.6 | 0.17 |
| Leptin (ng/ml)[b] | 1.7±3.0 | 1.4±2.9 | 1.7±3.0 | 0.42 | 11.5±2.1 | 11.2±2.2 | 11.5±1.9 | 0.92 |
| Total cholesterol (mmol/l) | 4.8±0.9 | 4.7±1.0 | 4.9±1.1 | 0.31 | 5.3±0.9 | 4.9±0.8 | 5.3±1.0 | 0.006 |
| LDL-cholesterol (mmol/l) | 3.0±0.9 | 2.9±0.9 | 3.1±1.0 | 0.35 | 3.1±0.8 | 2.7±0.8 | 2.9±0.8 | 0.012 |
| HDL-cholesterol (mmol/l) | 1.3±0.3 | 1.3±0.3 | 1.4±0.4 | 0.94 | 1.8±0.4 | 1.8±0.4 | 1.9±0.4 | 0.39 |
| Total/HDL-cholesterol ratio[b] | 3.6±1.4 | 3.5±1.3 | 3.7±1.4 | 0.67 | 3.0±1.3 | 2.8±1.3 | 2.9±1.3 | 0.34 |
| Triglycerides (mmol/l)[b] | 0.9±1.6 | 0.9±1.5 | 0.9±1.5 | 0.78 | 0.9±1.5 | 0.9±1.5 | 0.9±1.5 | 0.83 |
| NEFA (mmol/l) | 0.5±0.2 | 0.5±0.2 | 0.5±0.2 | 0.05 | 0.7±0.2 | 0.7±0.3 | 0.7±0.2 | 0.81 |

Data are expressed as mean ± SD. [a]Value calculated using standard linear regression, because convergence criteria could not be met using a random intercept model. [b]Geometric mean ± SD. HOMA = homeostasis model assessment. DZ = dizygotic, MZ DC = monozygotic dichorionic, MZ MC = monozygotic monochorionic.

**Table 2** Intra-pair correlations of MZ MC and MZ DC pairs, and of each sex by zygosity group before and after adjusting for covariates in the EFPTS.

| | Monozygotic | | Monozygotic | | Dizygotic | | | |
|---|---|---|---|---|---|---|---|---|
| Characteristic | MC | DC | Men | Women | Men | Women | OS | Covariates |
| n (of pairs) | 138 | 102 | 113 | 127 | 46 | 49 | 43 | |
| Body mass | 0.85/0.79 | 0.84/0.76 | 0.86/0.82 | 0.76/0.73 | 0.38/0.28 | 0.58/0.57 | 0.26/0.35 | Sex, age, height |
| BMI | 0.80/0.78 | 0.81/0.77 | 0.86/0.83 | 0.77/0.74 | 0.46/0.31 | 0.53/0.56 | 0.47/0.46 | Age |
| WHR | 0.87/0.69 | 0.88/0.71 | 0.79/0.74 | 0.70/0.66 | 0.39/0.28 | 0.44/0.48 | 0.31/0.15 | Sex, age |
| S4SF | 0.82/0.73 | 0.84/0.75 | 0.81/0.79 | 0.72/0.68 | 0.46/0.37 | 0.64/0.63 | 0.36/0.31 | Sex, age |
| Fat mass | 0.85/0.78 | 0.85/0.75 | 0.85/0.82 | 0.73/0.69 | 0.46/0.36 | 0.43/0.46 | 0.42/0.35 | Sex, age |
| Lean body mass | 0.93/0.81 | 0.93/0.79 | 0.86/0.82 | 0.79/0.78 | 0.43/0.39 | 0.65/0.58 | 0.25/0.39 | Sex, age, height |
| IGFBP1 | 0.49/0.39 | 0.60/0.53 | 0.55/0.51 | 0.45/0.43 | 0.31/0.21 | 0.08/0.06 | 0.12/-0.05 | Sex, age, BMI |
| Fasting insulin | 0.57/0.48 | 0.52/0.50 | 0.49/0.45 | 0.58/0.52 | 0.07/0.13 | 0.18/0.19 | 0.07/-0.01 | Age, S4SF |
| Insulin resistance | 0.54/0.47 | 0.53/0.50 | 0.49/0.46 | 0.57/0.51 | 0.03/0.08 | 0.14/0.17 | 0.04/-0.05 | Sex, age, S4SF |
| Fasting glucose | 0.74/0.66 | 0.73/0.67 | 0.65/0.65 | 0.70/0.69 | 0.28/0.24 | 0.57/0.60 | 0.31/0.32 | Sex, BMI |
| Beta cell function | 0.71/0.58 | 0.66/0.60 | 0.52/0.50 | 0.68/0.66 | 0.32/0.40 | 0.47/0.46 | 0.37/0.33 | Sex, age, S4SF |
| Leptin | 0.85/0.53 | 0.85/0.57 | 0.70/0.58 | 0.64/0.52 | 0.35/0.02 | 0.66/0.31 | 0.38/0.37 | Sex, age, S4SF |
| Total cholesterol | 0.76/0.74 | 0.77/0.72 | 0.78/0.74 | 0.73/0.73 | 0.52/0.51 | 0.51/0.44 | 0.63/0.51 | Age, S4SF |
| LDL-cholesterol | 0.78/0.79 | 0.81/0.74 | 0.81/0.79 | 0.77/0.75 | 0.52/0.51 | 0.59/0.52 | 0.68/0.58 | Sex, age, S4SF |
| HDL-cholesterol | 0.78/0.71 | 0.84/0.77 | 0.75/0.76 | 0.74/0.74 | 0.31/0.30 | 0.44/0.44 | 0.52/0.52 | Sex, S4SF |
| Total/HDL-cholesterol ratio | 0.81/0.78 | 0.86/0.81 | 0.84/0.82 | 0.78/0.76 | 0.50/0.41 | 0.50/0.49 | 0.65/0.54 | Age, WHR |
| Triglycerides | 0.58/0.56 | 0.67/0.60 | 0.59/0.54 | 0.63/0.60 | 0.29/0.41 | 0.34/0.34 | 0.16/0.17 | Sex, S4SF |
| NEFA | 0.49/0.35 | 0.43/0.39 | 0.39/0.38 | 0.34/0.37 | 0.10/0.14 | 0.20/0.18 | 0.25/0.26 | Sex, S4SF |

Values are unadjusted intra-pair correlation/adjusted intra-pair correlation. DC = dichorionic, MC = monochorionic, OS = opposite sex.

covariates, the AE model became the best fitting model. For IGFBP1, fasting insulin and insulin resistance, a DE model containing a non-additive genetic and unique environmental component had the best fit. The variation of the remaining blood parameters, including fasting glucose, beta cell function, leptin, HDL-cholesterol, total/HDL-cholesterol ratio, triglycerides and NEFA levels were best explained by an AE model (Table 3).
Quantitative sex differences were present in body mass, BMI, WHR, S4SF, fat mass, lean body mass, leptin and total/HDL-cholesterol ratio, because variance components estimates were significantly different between men and women (Table 3). The influences of additive genetic factors were larger in men than in women. For some traits, scalar sex differences were observed, implying that although variance components are equal across sexes, the total variances differ. As a result, total variances of IGFBP1, HDL-cholesterol and NEFA levels in women were larger than in men, but smaller for fasting glucose and HDL-cholesterol levels (Table 3).
After adjusting for covariates, quantitative sex differences remained significant only for body mass, BMI and fat mass (Table 3). In addition, scalar sex differences were significant for WHR, S4SF, lean body mass, IGFBP1, fasting glucose, leptin, HDL-cholesterol, total/HDL-cholesterol ratio and NEFA levels. Total variances of IGFBP1, HDL-cholesterol and NEFA levels were larger in women than in men, but smaller for WHR, S4SF, lean body mass, fasting glucose, leptin and total/HDL-cholesterol ratio (Table 3).
The adjusted correlations of WHR, fasting insulin and triglycerides levels for opposite-sex pairs were smaller than the correlations for like-sexed DZ pairs, suggesting that different genetic factors influence these traits in men and women (Table 2). However, the correlations between the additive genetic factors in opposite-sex pairs were not significantly smaller than 0.5 ($p$>0.05).
Broad-sense heritability estimates (encompassing both additive and non-additive genetic effects) were slightly lower after adjusting for covariates, with the exception of total cholesterol and LDL-cholesterol, which had a higher heritability after adjustment (Table 3).
In summary, heritability estimates of body mass, BMI and fat mass were 84, 85 and 81% for men and 74, 75 and 70% for women, respectively. WHR, S4SF and lean body mass had heritability estimates of 70, 74 and 81%, correspondingly. For fasting glucose, fasting insulin, insulin resistance, beta cell function and IGFBP1 levels, genetic factors explained 67, 49, 48, 62 and 47% of the total variation, respectively. Finally, heritability estimates of total cholesterol, triglycerides, HDL-cholesterol, LDL-cholesterol, total/HDL-cholesterol ratio, NEFA and leptin levels were 75, 58, 76, 78, 79, 37 and 53%, respectively (Table 3).

## 2.5 DISCUSSION

To determine the genetic contribution to T2D, we estimated the heritabilities of 18 anthropometric and metabolic characteristics related to this disease, including parameters quantifying obesity, glucose intolerance/insulin resistance and dyslipidaemia. The traits were measured in 138 DZ and 240 MZ young healthy twin pairs recruited from the population-based EFPTS.[36]
Obesity, defined as excess accumulation of adipose tissue, is a complicated trait to measure and numerous methods exist for its determination.[41] In the present study several parameters defining obesity, including body mass, BMI, S4SF, WHR and fat mass were used and

**Table 3** Variance components estimates and 95% confidence intervals of best-fitting models expressed in percentages.

| | Unadjusted | | | | | | | Adjusted | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Characteristic | Model | | $a^2(H^2)$ | $c^2$ | $d^2(H^2)$ | $e^2$ | S | Model | | $a^2(H^2)$ | $c^2$ | $d^2(H^2)$ | $e^2$ | S |
| Body mass | AE | M | 90 (86-93) | - | - | 10 (7-14) | | AE | M | 84 (78-88) | - | - | 16 (12-22) | |
| | AE | W | 80 (73-85) | - | - | 20 (15-27) | | AE | W | 74 (66-80) | - | - | 26 (20-34) | |
| BMI | AE | M | 87 (82-90) | - | - | 13 (10-18) | | AE | M | 85 (79-89) | - | - | 15 (11-21) | |
| | AE | W | 76 (68-82) | - | - | 24 (18-32) | | AE | W | 75 (67-81) | - | - | 25 (19-33) | |
| WHR | AE | M | 94 (92-96) | - | - | 6 (4-8) | | AE | | 70 (63-75) | - | - | 30 (25-37) | M>W |
| | AE | W | 70 (59-77) | - | - | 30 (23-41) | | | | | | | | |
| S4SF | AE | M | 88 (83-91) | - | - | 12 (9-17) | | AE | | 74 (68-79) | - | - | 26 (21-32) | M>W |
| | AE | W | 75 (67-81) | - | - | 25 (19-33) | | | | | | | | |
| Fat mass | AE | M | 88 (84-92) | - | - | 12 (8-16) | | AE | M | 81 (74-86) | - | - | 19 (14-26) | |
| | AE | W | 74 (65–80) | - | - | 26 (20-35) | | AE | W | 70 (60-77) | - | - | 30 (23-40) | |
| Lean body mass | AE | M | 97 (96-98) | - | - | 3 (2-4) | | AE | | 81 (76-84) | - | - | 19 (16-24) | M>W |
| | AE | W | 82 (76-87) | - | - | 18 (13-24) | | | | | | | | |
| IGFBP1 | DE | | - | - | 56 (47-64) | 44 (36-53) | W>M | DE | | - | - | 47 (36-56) | 53 (44-64) | W>M |
| Fasting insulin | DE | | - | - | 54 (45-62) | 46 (38-55) | | DE | | - | - | 49 (39-58) | 51 (42-61) | |
| Insulin resistance | DE | | - | - | 52 (42-60) | 48 (40-58) | | DE | | - | - | 48 (38-57) | 52 (43-62) | |
| Fasting glucose | AE | | 72 (66-77) | - | - | 28 (23-34) | M>W | AE | | 67 (60-73) | - | - | 33 (27-40) | M>W |
| Beta cell function | AE | | 69 (63-75) | - | - | 31 (25-37) | | AE | | 62 (54-69) | - | - | 38 (31-46) | |
| Leptin | AE | M | 92 (88-94) | - | - | 8 (6-12) | | AE | | 53 (44-61) | - | - | 47 (39-56) | M>W |
| | AE | W | 63 (52-72) | - | - | 37 (28-48) | | | | | | | | |
| Total cholesterol | ACE | | 49 (28-74) | 29 (4-48) | - | 22 (18-28) | | AE | | 75 (69-79) | - | - | 25 (21-31) | |
| LDL-cholesterol | ACE | | 43 (24-67) | 37 (14-54) | - | 20 (16-25) | M>W | AE | | 78 (73-82) | - | - | 22 (18-27) | |
| HDL-cholesterol | AE | | 82 (78-86) | - | - | 18 (14-22) | W>M | AE | | 76 (70-81) | - | - | 24 (19-30) | W>M |
| Total/HDL- | AE | M | 88 (83-91) | - | - | 12 (9-17) | | AE | | 79 (74-83) | - | - | 21 (17-26) | M>W |
| cholesterol ratio | AE | W | 79 (72-85) | - | - | 21 (15-28) | | | | | | | | |
| Triglycerides | AE | | 61 (52-68) | - | - | 39 (32-48) | | AE | | 58 (49-66) | - | - | 42 (34-51) | |
| NEFA | AE | | 46 (36-54) | - | - | 54 (46-64) | W>M | AE | | 37 (25-47) | - | - | 63 (53-75) | W>M |

$a^2$ = additive genetic variance, $c^2$ = common environmental variance, $d^2$ = non-additive genetic variance, $e^2$ = unique environmental variance, $H^2$ = broad heritability, M = men, S = scalar effect, W = women.

heritability estimates ranged from 70 to 85%. According to Table S1, the heritabilities identified by us for BMI and body mass are in agreement with those reported in other twin studies, but the heritability identified by us for WHR is somewhat higher.[7,15,18,42] Heritability estimates for S4SF and fat mass are not often reported and therefore we were not able to make a valid comparison. Despite the variety of measurements used to quantify obesity, the heritabilities of the different measurements were consistently in a high range, indicating that the contribution of genetic factors to total phenotypic variation in obesity is of great importance in the studied twin sample.

Quantitative sex differences were significant for body mass, BMI and fat mass, with heritability estimates of 84, 85 and 81% in men and 74, 75 and 70% in women, respectively. Our results confirm those from other twin studies reporting quantitative sex differences in the heritability of body mass and BMI.[4-6,9,20,43] However, in other twin studies the highest heritability estimates were not always observed in men (Table S1), indicating that the influence of environmental factors in the total variation of these traits according to sex may vary in different populations.

In the present study, 49% of the total variation of fasting insulin was explained by genetic factors, which is in agreement with other twin studies (Table S1)[44,45] The heritability of fasting glucose ($H^2$=67%) was higher than that observed in other twin studies, where it ranged between 12 and 58% (Table S1).[45,46] The heritabilities of beta cell function ($H^2$=62%) and insulin resistance ($H^2$=48%) were not higher than those of fasting glucose ($H^2$=67%) and fasting insulin levels ($H^2$=49%). This indicates that no additional power was obtained by calculating these alternative phenotypic markers with the homeostasis model assessment.

The variation of total cholesterol, triglycerides, HDL-cholesterol, LDL-cholesterol and total/HDL-cholesterol ratio was primarily explained by additive genetic factors, accounting for 75, 58, 76, 78 and 79% of the total variance, respectively. No evidence was observed for quantitative sex differences in heritability estimates for these traits. The estimates are slightly higher than the heritability estimates reported in the literature, but are in the same high range (Table S1). To the best of our knowledge, ours is the first large twin study reporting the heritability of NEFA levels, which was 37%. This is somewhat surprising, since excessive circulating fatty acids represent a major contributor to the development of insulin resistance.[47] The heritability determined for NEFA levels in the current study was low compared with those reported for the other blood lipid parameters, indicating that environmental factors, e.g. nutrition, may be more important in determining the variation of NEFA levels than genetic factors.

Additive genetic variance explained 53% of the variation of total leptin concentration. In other western adult twin studies, heritabilities of total leptin concentrations ranged from 34 to 55%,[48,49] which is in agreement with our findings. Compared with other traits, the correlations and heritability estimates of leptin were strongly reduced after adjusting for covariates, which is probably caused by the adjustment for fat (using S4SF). This is not very surprising, since leptin is synthesised in and secreted from adipose tissue, and plasma leptin levels are increased in obese humans in direct proportion to body fat mass.[35] The heritability of IGFBP1 levels ($H^2$=47%) determined in our study was also relatively high, since the heritability in two other twin studies ranged from 0 to 36%.[44,50] Interestingly, the heritabilities of IGFBP1 levels in the latter studies were determined in middle-aged twins (mean age of 63 years). This might suggest that the heritability of IGFBP1 decreases with increasing age. Longitudinal twin studies need to be carried out to verify this observation.

We are the first to report that a model containing non-additive genetic factors is the best-fitting model for insulin resistance, fasting insulin and IGFBP1 levels.[7,18,22,24,44-46] In addition to the twin model fitting procedure, twin correlations can also give an impression whether non-additive genetic effects might be involved i.e. a DZ correlation of less than one-half of the MZ correlation suggests a contribution of non-additive genetic effects.[18,44-46] The smaller sample size of some other studies could be responsible for the fact that a DE model was not observed. Furthermore, the presence of non-additive genetic effects were not always tested. However, when non-additive genetic effects are observed, it implies that part of the variance is explained by interactions between alleles at the same locus or on different loci. Although this observation is likely to be the result of a lack of power, we cannot exclude real non-additive genetic effects in the absence of additive genetic effects, as these three traits are closely related to or are a direct gene product.

MZ MC twins have a more adverse intra-uterine environment than MZ DC and DZ twins, resulting in a significantly lower weight at birth and higher perinatal mortality and morbidity.[36] Hence, it has been hypothesised that for disorders like T2D, in the development of which prenatal environment plays a role, the classical twin study might be an unreliable method of estimating heritabilities.[51,52] In the current twin sample, intra-pair correlations did not differ between MZ MC and MZ DC twins, suggesting that the chorion type of MZ twins did not bias the heritability estimates of the studied traits.

With the exception of NEFA levels, the genetic contribution to the traits studied in our twin sample was high. When comparing the heritability values found by us with those reported in other twin studies (Table S1), our heritability estimates are in a slightly higher range. An important aspect affecting heritability estimates is the accuracy of the zygosity determination used in the present study via sequential analysis based on sex, fetal membranes, umbilical cord blood groups, placental alkaline phosphatase and DNA marker analysis. In other twin studies, zygosity was often determined using questionnaires,[4] which sometimes leads to misclassification and consequently to underestimation of heritability.[53] Furthermore, the homogeneous composition of the studied sample is likely to contribute to the high heritability estimates of our study, as all twins included were born in the Belgian province of East Flanders, which is a relatively small but rather densely populated area.[36] Another factor possibly contributing to the high heritability estimates is age, as shown by the fact that several large twin studies have reported a decrease in the heritability of obesity (BMI) in adults with increasing age.[5,12,16] The average age of our twin sample is young (mean = 25 years) and the age range is small (18–34 years). As the power to detect quantitative trait loci is positively correlated with the size of the genetic effect,[3] our study suggests that the search for candidate genes might be more efficient in a young homogeneous population.

In conclusion, this study is the first large study to give a comprehensive overview of the heritabilities of multiple risk factors related to T2D within the same twin sample. The variation of traits related to obesity, glucose intolerance/insulin resistance and dyslipidaemia in this Belgian twin sample is in general highly explained by genetic factors. These high heritabilities are very promising for follow-up to this research, in which, in order to explain some of the genetic variance observed, sib-pair linkage analyses will be performed using microsatellite markers located in the close vicinity of selected candidate genes.

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## 2.8 SUPPLEMENTARY MATERIAL

**Table S1.** Twin studies (>200 twin pairs) estimating heritabilities ($H^2$, expressed in percentage) of obesity parameters, fasting glucose, fasting insulin and lipid levels in adults.

| Reference | Registry | Pairs (*n*) | Age (years) | | Adjustment | Sex | $H^2$ | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Mean | Range | | | BM | BMI | WHR | S4SF | FATM | FG | FI | TC | HDL | LDL | TRG |
| [4] | Eight registries[a] | 36295[b] | – | 20–39 | Age | M | – | 45–84 | – | – | – | – | – | – | – | – | – |
| | | | | | Age | W | – | 64–85 | – | – | – | – | – | – | – | – | – |
| [5] | Virginia | 5588 | – | – | – | M | – | 72 | – | – | – | – | – | – | – | – | – |
| | | | | | – | W | – | 75 | – | – | – | – | – | – | – | – | – |
| [5] | Australia | 3569[b] | – | – | – | M | – | 70–80 | – | – | – | – | – | – | – | – | – |
| | | | | | – | W | – | 69–78 | – | – | – | – | – | – | – | – | – |
| [6] | Danish | 1233[b] | – | 46–76 | Age, zygosity | M | – | 46–61 | – | – | – | – | – | – | – | – | – |
| | | | | | Age, zygosity | W | – | 75–77 | – | – | – | – | – | – | – | – | – |
| [7] | Danish | 303 | 67 | 55–74 | – | M | – | 58 | 22 | – | – | – | 36 | – | 56 | – | 50 |
| | | | | | – | W | – | 90 | 10 | – | – | – | 14 | – | 84 | – | 34 |
| [8] | Danish | 624 | 38 | 18–67 | Age | M | – | 63 | – | 65 | 63 | – | – | – | – | – | – |
| | | | | | Age | W | – | 58 | – | 61 | 59 | – | – | – | – | – | – |
| [9] | Finnish | 7245 | 31 | 18–54 | Age | M | – | 72 | – | – | – | – | – | – | – | – | – |
| | | | | | Age | W | – | 68 | – | – | – | – | – | – | – | – | – |
| [10c] | KPWTS | 434 | 42 | – | Age | W | – | 89 | – | – | – | – | – | – | 68 | 98 | 80 |
| | | | | | Age, environment, BMI | W | – | – | – | – | – | – | – | – | 69 | 91 | 51 |
| [11] | KPWTS | 315[b] | – | 18–91 | – | W | – | 79–80 | – | – | – | – | – | – | – | – | – |
| [12] | Minnesota | 1033[b] | – | 18–81 | Sex | M/W | 70–86 | 63–82 | – | – | – | – | – | – | – | – | – |
| [13c] | NAS-NRC | 514 | – | 42–56 | – | M | 56 | 64 | – | – | – | – | – | 43 | 46 | 57 | 56 |
| [14c] | NAS-NRC | 4071[b] | – | 15–53 | – | M | 78–81 | 77–84 | – | – | – | – | – | – | – | – | – |
| [15c] | NAS-NRC | 265 | – | 59–70 | BMI | M | – | 63 | 31 | – | – | – | – | – | – | – | – |
| [16] | NAS-NRC | 243[b] | – | 20–63 | – | M | – | 73–82 | – | – | – | – | – | – | – | – | – |
| [17] | Swedish | 289 | 66 | 52–86 | Sex, age | M/W | – | 52 | – | – | – | – | – | – | 54 | – | 43 |
| [18] | Swedish | 318 | 65 | 45–85 | Age | M | – | 58 | 28 | – | – | – | 27 | – | – | – | – |
| | | | | | Age | W | – | 73 | 49 | – | – | – | 49 | – | – | – | – |
| [19] | Swedish | 673 | 59 | – | Age | M | – | 74 | – | – | – | – | – | – | – | – | – |
| | | | | | Age | W | – | 69 | – | – | – | – | – | – | – | – | – |
| [20] | Norwegian | 2570 | – | 18–25 | – | M | – | 71 | – | – | – | – | – | – | – | – | – |
| | | | | | – | W | – | 79 | – | – | – | – | – | – | – | – | – |

| Reference | Registry | Pairs (*n*) | Age (years) Mean | Range | Adjustment | Sex | $H^2$ BM | BMI | WHR | S4SF | FATM | FG | FI | TC | HDL | LDL | TRG |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| [21] | German | 222 | 34 | – | – | M/W | 89 | 97 | – | – | – | – | – | 64 | 59 | 66 | 72 |
| [22] | Danish | 607 | 38 | 18–67 | Age | M | – | – | – | – | – | 38 | 37 | – | – | – | – |
| | | | | | Age | W | – | – | – | – | – | 12 | 54 | – | – | – | – |
| [23] | Dutch | 209 | 44 | 34–63 | Sex, assay batch, truncation | M/W | – | – | – | – | – | 50 | 20–25 | – | – | – | – |
| [24c] | KPWTS | 278 | 51 | 30–91 | Age, behaviour, BMI | W | – | – | – | – | – | – | 54 | – | – | – | – |
| [25] | Three registries[d] | 1859[b] | – | 28–92 | Sex, age | M/W | – | – | – | – | – | – | – | 57–77 | 62–72 | 61–77 | 48–62 |
| [26] | Australia | 205 | 23 | 18–34 | – | M | – | – | – | – | – | – | – | 54 | 24 | – | 53 |
| | | | | | – | W | – | – | – | – | – | – | – | 54 | 24 | – | 51 |
| [27] | Dutch | 203 | 44 | 34–63 | Sex, age | M/W | – | – | – | – | – | – | – | 68 | 71 | 69 | 59 |
| [28] | KPWTS | 348[b] | 41,51 | – | Age | W | – | – | – | – | – | – | – | 63–66 | 73 | 65 | 50–65 |
| [29] | St Thomas', UK | 1733 | 48 | 18–79 | Age, fas, HRT, MPS | W | – | – | – | – | – | – | – | 42 | 42 | 46 | 63 |
| [30] | Swedish | 302[b] | 66 | 52–86 | Sex , age | M/W | – | – | – | – | – | – | – | 32–63 | 55–76 | – | 28–72 |
| [31] | Swedish | 725[b] | – | 17–85 | Med, fas, dia, sex, age | M/W | – | – | – | – | – | – | – | 49–65 | – | – | 19–62 |
| [32] | Pittsburgh | 204 | 21 | 18–30 | Age, sex, eth, BMI, nut | M/W | – | – | – | – | – | – | – | 68 | 67 | 78 | 51 |
| [33] | Australia | 208[b] | – | 18–47 | Sex, age | M/W | – | – | – | – | – | – | – | 50–83 | – | – | – |
| [34] | Danish | 756 | – | – | Age | M | – | – | – | – | – | – | – | 74 | 34 | 71 | 0 |
| | | | | | Age | W | – | – | – | – | – | – | – | 74 | 54 | 71 | 60 |
| [42] | KPWTS | 340 | 52 | 31–90 | Age | W | – | – | 52 | – | – | – | – | – | – | – | – |
| [43] | Minnesota | 4020 | 40 | 28–52 | Age | M | 61 | – | – | – | – | – | – | – | – | – | – |
| | | | | | Age | W | 73 | – | – | – | – | – | – | – | – | – | – |
| Present study | Belgian (EFPTS) | 378 | 25 | 18–34 | See Table 3 | M | 84 | 85 | 70 | 74 | 81 | 67 | 49 | 75 | 76 | 78 | 58 |
| | | | | | | W | 74 | 75 | 70 | 74 | 70 | 67 | 49 | 75 | 76 | 78 | 58 |

[a]Australian, Danish, Finnish, Italian, Dutch, Norwegian, Swedish and the St Thomas' UK twin registry. [b]In these studies heritabilities between different age groups are compared; in the table the range is presented. [c]Classical heritability estimates: calculated as twice the difference of the MZ and DZ intra-class correlations. [d]Australian, Swedish and the Dutch twin registry. Behav = behaviour, BM = body mass, dia = diabetes, env = environment, eth = ethnicity, fas = fasting, FATM = fat mass, FG = fasting glucose, FI = fasting insulin, HRT = hormone replacement therapy, KPWTS = Kaiser-Permanente Women Twins Study, Med = medication, MPS = menopausal status, NAS-NRC = National Academy of Sciences – National Research Council, nut = nutrition, S4SF = sum of four skinfold thicknesses, TC= total cholesterol, TRG = triglycerides, (–) = not determined or unknown.

# CHAPTER 3

# ANTHROPOMETRY, CARBOHYDRATE AND LIPID METABOLISM IN THE EAST FLANDERS PROSPECTIVE TWIN SURVEY: LINKAGE OF CANDIDATE GENES USING TWO SIB-PAIR BASED VARIANCE COMPONENTS ANALYSES

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## 3.1 ABSTRACT

Insulin resistance and obesity are underlying causes of type 2 diabetes (T2D) and therefore much interest is focused on the potential genes involved. A series of anthropometric and metabolic characteristics were measured in 240 MZ and 112 DZ twin pairs recruited from the East Flanders Prospective Twin Survey. Microsatellite markers located close to *ABCC8*, *ADIPOQ*, *GCK*, *IGF1*, *IGFBP1*, *INSR*, *LEP*, *LEPR*, *PPARγ* and the *RETN* gene were genotyped. Univariate single point variance components linkage analyses were performed using two methods: 1) the standard method, only comprising the phenotypic and genotypic data of the DZ twin pairs and 2) the extended method, also incorporating the phenotypic data of the MZ twin pairs. Suggestive linkages (LOD>1) were observed between the *ABCC8* marker and waist-to-hip ratio (WHR) and HDL-cholesterol levels. Both markers flanking *ADIPOQ* showed suggestive linkage with triglycerides levels, the upstream marker also with body mass and HDL-cholesterol levels. The *IGFBP1* marker showed suggestive linkage with fat mass, fasting insulin and leptin levels and the *LEP* marker showed suggestive linkage with birth weight. This study suggests that DNA variants in the *ABCC8*, *ADIPOQ*, *IGFBP1* and *LEP* gene region may predispose to T2D. In addition, the two methods used to perform linkage analyses yielded similar results. This was however not the case for birth weight where chorionicity seems to be an important confounder.

## 3.2 INTRODUCTION

T2D is a complex disorder that represents a major international public health threat.[1] Although lifestyle factors play a prominent role in the development of the disease, the high prevalence of T2D in specific ethnic groups and the much higher concordance rates of traits associated with the disorder in monozygotic (MZ) twins compared to dizygotic (DZ) twins indicate that genetic predisposition is also important.[2,3] To uncover genes involved in T2D, numerous genome-wide linkage scans have been carried out and evidence for linkage at several chromosomal regions has been reported.[4-6] Usually, these regions are large and contain many genes and therefore candidate genes in subsequent studies are often selected based on pre-existing knowledge. Currently, much interest is focused on genes involved in insulin resistance and obesity, since these two intertwined factors are considered to be underlying causes of T2D.[7,8]

In the condition of insulin resistance, normal insulin concentrations fail to trigger a normal metabolic response. To resist diabetes, pancreatic beta cells then need to overcompensate with sufficient insulin release.[9] Pancreatic beta cells produce and secrete insulin mainly in response to blood glucose, which is taken up into the cells through the glucose transporter isotype 2 (GLUT2) and subsequently phosphorylated into glucose-6-phosphate by glucokinase (GCK).[9,10] This metabolic activity in the pancreatic beta cell leads to an increase in the ATP/ADP ratio and closure of the ATP-sensitive potassium ($K_{ATP}$) channel, which is composed of the inward rectifying $K^+$ channel (Kir6.2) and the sulfonylurea receptor (SUR1; encoded by *ABCC8*). The closure is followed by membrane depolarisation, which activates voltage gated calcium channels and leads to intracellular $Ca^{2+}$ rise triggering insulin secretion.[11,12]

In addition to insulin there are also other hormones with insulin-like effects. The most important of these is insulin-like growth factor 1 (IGF1), which is the second most powerful peptide with glucose lowering effects.[13] The effects of insulin and IGF1 on carbohydrate, lipid and protein metabolism are mediated by their receptors (INSR, IGF1R). Insulin and IGF1 are able to bind to each others receptor, although the affinity is considerably lower.[14] Circulating IGF1 is almost entirely bound to one of the six members of the IGF binding proteins (IGFBPs) family. Although the majority of IGF1 is bound to IGFBP3, IGFBP1 has been suggested to be the short-term regulator of IGF1 bioactivity and is negatively associated with risk factors of cardiovascular disease and positively with insulin sensitivity.[13,15,16]
Obesity, another major factor underlying T2D, is defined as excess accumulation of adipose tissue. Adipose tissue, formerly considered to be an inert energy depository, is now also known as an important endocrine organ producing active molecules called adipocytokines including adiponectin, leptin and resistin, which are encoded by *ADIPOQ*, *LEP* and *RETN*, respectively.[17] Increased adiponectin levels are associated with weight loss and increased insulin sensitivity.[18,19] Additionally, in prepubertal children, adiponectin is negatively associated with plasma lipid markers, except for HDL-cholesterol that shows a positive relation.[17] Leptin, which biological activities are carried out through selective binding to its receptor (LEPR), is mainly produced by adipocytes, but also expressed in other tissues including placenta, ovaries, skeletal muscle, stomach, pituitary and liver.[19,20] Leptin acts as a satiety signal affecting central circuits in the hypothalamus resulting in suppressed food intake and increased energy expenditure.[19] Furthermore peripheral leptin seems to play a role in controlling cellular lipid balance, glucose homeostasis and insulin sensitivity.[19,21] Resistin has been linked to insulin resistance in murine models.[17,19] In humans, however, the relationship between resistin and markers of the metabolic syndrome is still controversial.[18,19,22] Besides secreting active molecules adipose tissue has the capacity to expand drastically during life,[14] which is correlated with many unfavourable side effects. Therefore the role of proteins such as the peroxisome proliferator-activated receptor $\gamma$ (PPAR$\gamma$) and IGF1, which among other processes regulate fat cell formation and functioning, is also of great interest in the pathology of obesity.[14,23]

Previously we estimated the heritability of 18 anthropometric and metabolic characteristics associated with T2D measured in 378 healthy twin pairs recruited from the East Flanders Prospective Twin Survey (EFPTS).[3] In general, heritability estimates of the traits studied in our twin sample were high. In order to explain some of the genetic variance observed, we performed univariate single point variance components linkage analyses using microsatellite markers located within or near candidate genes including *ABCC8*, *ADIPOQ*, *GCK*, *IGF1*, *IGFBP1*, *INSR*, *LEP*, *LEPR*, *PPAR$\gamma$* and *RETN*. Linkage to metabolic traits in these candidate gene regions provides evidence for the possible presence of genetic variation in that region influencing these traits and is an incentive for further fine mapping the region. Several software packages are available to carry out sib-pair based variance components linkage analyses. The statistical package Mx is especially designed to analyse twin data,[24] but another commonly used program is MERLIN.[25] The advantage of Mx compared to MERLIN is that Mx also incorporates the phenotypic data of the MZ twins. This allows to partition the total variation into effects due to the quantitative trait locus (QTL) (Q), additive genetic (A), non-additive genetic (D) or common environmental (C), and unique environmental effects (E), resulting in a very accurate estimation of the variance components. In addition, when

the distribution of a trait differs among men and women it is possible to model these sex differences by using a scalar model. By comparing the fit-functions of the different models one can assess which model provides the best fit to the data and use that model to conduct the linkage analysis. MERLIN, on the contrary simply incorporates the phenotypic and genotypic data of the DZ twins and decomposes the total phenotypic variance into effects due to the QTL (Q), additive genetic factors (A) and unique environmental factors (E). In the present study, sib-pair variance component linkage analyses were conducted using both the "MERLIN method", which we call the standard method, and the "Mx method", which we call the extended method.

## 3.3 MATERIALS AND METHODS

### *3.3.1 Participants*

The EFPTS is a population-based twin register, which started in 1964 and has been recording all multiple births in the Belgian Province of East Flanders until the present.[26] For the current study, a series of anthropometric and metabolic phenotypes of 378 complete twin pairs were available, divided over 138 DZ pairs (46 male, 49 female and 43 mixed pairs) and 240 MZ pairs (113 male and 127 female pairs). Since not all participants were willing to provide DNA, DNA was available from 288 complete twin pairs comprising 112 DZ pairs (34 male, 43 female and 35 mixed pairs) and 176 MZ pairs (78 male and 98 female pairs). In the present analysis, if genotypic data were absent then the phenotypic data could only be used if the twins were MZ. As a result, in the current analysis phenotypic data of 352 complete pairs were used, divided over 112 DZ pairs and 240 MZ pairs, of which 138 pairs were monochorionic (MC) and 102 were dichorionic (DC). Birth weights were obtained from obstetric records, and gestational age reported by the obstetrician was calculated as the number of completed weeks of pregnancy based on the last menstrual period. The methods used to measure the phenotypes at adult age and a detailed description of the twin sample has been described earlier.[3,26] The Ethics Committee of the Faculty of Medicine of the Katholieke Universiteit Leuven approved the project and all participants gave informed consent.

### *3.3.2 DNA extractions and marker selection*

Genomic DNA was initially extracted from available placental tissue collected at birth and/or twins were contacted and whole blood or mouth swabs were taken for DNA extraction. The DNA mini kit (Qiagen) was used for placental and mouth swab DNA extraction, the Wizard kit (Promega) was used for blood DNA extraction, both according to manufacturer's instructions. Microsatellite markers (di- and tetranucleotide repeats) were selected from the human UniSTS map (www.ncbi.nlm.nih.gov/genome/sts). Markers were chosen based on distance to the candidate gene and level of heterozygosity. The location of the markers and candidate genes as well as marker heterozygosity is presented in Table 1.

**Table 1**. Gene and marker positioning, primer sequences and PCR conditions.

| *Gene* | Marker | Physical Position[a] | Genetic[b] | Forward primer sequence (5′→3′)[c] | Reverse primer sequence (5′→3′) | Product[d] | C | T | $MgCl_2$[e] | Alleles | H |
|---|---|---|---|---|---|---|---|---|---|---|---|
| *ABCC8* | | 17.371.009-17.455.025 | | | | | | | | | |
| | D11S902 | 17.445.082-17.445.228 | 21.5 | **HEX**-CGGCTGTGAATATACTTAATGC | CAACAGCAATGGGAAGTT | 137-159 | 33 | 55 | 2.25 | 12 | 82 |
| | D3S1602 | 187.514.441-187.514.733 | 201.1 | **TET**-AGAGCCTTCTATGGGTCTACAT | AGCTCAACCTTCAAACATACATT | 271-297 | 31 | 55 | 2.25 | 13 | 84 |
| *ADIPOQ* | | 188.043.157-188.058.946 | | | | | | | | | |
| | D3S3686 | Not available | 203.8 | **HEX**-AGGGTATTTCATTCCCATTG | CCAGGTTACGCCAAGTG | 105-135 | 33 | 55 | 2.25 | 15 | 81 |
| | GCK | 44.142.647-44.142.841 | 67.4 | **TET**-CACACCAAAACTGCCTGTATTAG | TTGGTCAGTGTAGGCTGAACTC | 194-204 | 31 | 55 | 2.25 | 5 | 49 |
| *GCK* | | 44.150.395-44.195.563 | | | | | | | | | |
| | D7S2427 | 44.931.275-44.931.510 | 68.5 | **TET**-AGGATTGTTTGAGCCCAGA | TTAGATTCCCATCAGCAGC | 207-245 | 31 | 55 | 2.25 | 17 | 82 |
| *IGF1* | | 101.313.806-101.398.454 | | | | | | | | | |
| | IGF1 | 101.399.183-101.399.376 | 109.5 | **FAM**-GCTAGCCAGCTGGTGTTATT | ACCACTCTGGGAGAAGGGTA | 174-196 | 35 | 60 | 2.25 | 7 | 53 |
| | D7S478 | 44.898.728-44.898.851 | 69.0 | **HEX**-TGTGTCATTACGCTTTTCATC | TCAAATGGTTCAGGAGAAAGA | 108-130 | 40 | 60 | 2.25 | 10 | 70 |
| *IGFBP1* | | 45.894.484-45.899.792 | | | | | | | | | |
| | D7S2506 | 47.646.979-47.647.146 | 69.6 | **TET**-CAGCAGGGCTTGAAATGAAC | ACACAGTGGAGCTGGCATAG | 150-186 | 35 | 60 | 1.69 | 18 | 84 |
| | D19S1034 | 6.064.256-6.064.482 | 20.8 | **FAM**-AGGCTGTGGTGAGCTATGAC | GTGTCCCTAGCACCTAGCAA | 217-239 | 35 | 55 | 1.5 | 7 | 70 |
| *INSR* | | 7.067.638-7.245.011 | | | | | | | | | |
| *LEP* | | 127.668.567-127.684.917 | | | | | | | | | |
| | D7S530 | 128.989.684-128.989.791 | 134.6 | **FAM**-TGCATTTTAGTGGAGCACAG | CAGGCATTGGGAACTTTG | 98-118 | 32 | 55 | 2.25 | 11 | 76 |
| | D1S2638 | 63.325.557-63.325.776 | 96.0 | **HEX**-CTTGGATTGGTGGGTACTA | TGAGGTTTCAGGGTGGCT | 223-245 | 33 | 55 | 2.25 | 12 | 82 |
| *LEPR* | | 65.658.906-65.875.410 | | | | | | | | | |
| | D1S198 | 66.783.773-66.784.080 | 99.3 | **TET**-GACTTCACCATCAACGCCTG | CAGGAAAGTGGATGTGACGA | 307-325 | 31 | 55 | 2.25 | 10 | 81 |
| | D3S1263 | 11.492.252-11.492.482 | 36.1 | **FAM**-CTGTTGACCCATTGATACCC | GATGCCTTGCGGCTATGA | 253-297 | 32 | 55 | 2.25 | 19 | 87 |
| *PPARγ* | | 12.304.359-12.450.843 | | | | | | | | | |
| *RETN* | | 7.639.972-7.641.340 | | | | | | | | | |
| | D19S413 | 9.040.462-9.040.546 | 32.4 | **TET**-GTTTATTTTAAATGCTCTTACCACA | CCATCAACTCACCTACTTATC | 65-85 | 31 | 55 | 2.25 | 11 | 79 |

C = cycles, H = heterozygosity (%), T = annealing temperature (°C ). [a]Physical map positions (bp) are according to the sequence map accessible on the NCBI website (http://www.ncbi.nlm.nih.gov/mapview/map_search.cgi?taxid=9606 and http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=gene). [b]Genetic map positions (cM) are according to Marshfield. [c]Forward primers were 5′-labeled with fluorescent dyes (in bold). [d]Range product size in bp. [e]$MgCl_2$ in mM.

### 3.3.3 Polymerase chain reactions (PCRs)

10 PCR protocols were used to amplify 14 markers of 10 candidate genes. Six markers could be optimised to fit in two multiplexed PCR protocols (multiplex 1: markers D3S1602, GCK, D7S2427 and D1S198; multiplex 2: markers D3S3686 and D11S902) and the others were amplified in singleplex PCR protocols. Primer sequences including fluorescent labels, $MgCl_2$ concentrations, PCR annealing temperature and the number of cycles are listed in Table 1. Further PCR ingredients (15 or 20 µl) consisted of 1x PCR buffer (Invitrogen, Breda, the Netherlands), 0.33 mM dNTPs (GE Healthcare, Eindhoven, the Netherlands), 4-8 pmol primers (Eurogentec, Seraing, Belgium), 5% DMSO and 0.5-1 U *Taq* DNA polymerase (Invitrogen). All PCRs were performed in Biometra T1 thermal cyclers (Westburg, Leusden, the Netherlands), starting with an initial denaturation at 95°C for 5 min and ending with a final extension of 10 min at 72°C.

### 3.3.4 Genotyping

All TET-labelled PCR products were diluted 10 times, FAM-labelled products 5-10 times and HEX-labelled products 0-5 times. Labelled fragments were size-resolved by capillary electrophoresis on the ABI3100 Genetic Analyzer and size calling was performed with Genescan Software version 3.7 (Applied Biosystems, Nieuwerkerk a/d IJssel, the Netherlands). The TAMRA 350-size standard was used for fragment length calling. Two experienced researchers scored the final genotypes independently and a third expert was consulted for discrepant results.

### 3.3.5 General statistical analysis

Anthropometric and metabolic characteristics are expressed as mean $\pm$ SD. Traits with a skewed distribution were converted to a normal distribution by log transformation. Differences between men and women according to zygosity were tested using the PROC MIXED method, in which adjustment for the intra-twin pair relationship is possible. Differences were considered significant if $p<0.05$. For all traits effects of potential covariates were also checked using PROC MIXED, which has been described in detail before.[3] General statistical analyses were conducted using the statistical package SAS (version 9.1, SAS Institute).

### 3.3.6 Linkage analysis

A $\chi^2$-test was used to check Hardy-Weinberg equilibrium for genotype frequencies of the microsatellite markers, using only one randomly selected co-twin per pair (using genotypes of MZ and DZ twin pairs). To test for linkage between marker loci and the anthropometric and metabolic characteristics, univariate single point variance components linkage analyses were performed using the statistical package Mx.[24] The script was downloaded from the GenomEUtwin Mx-script library (http://www.psy.vu.nl/mxbib/). Linkage analysis was performed with and without adjusting for significant covariates. In addition we compared two methods: (1) standard sib-pair variance components linkage analysis, only comprising the phenotypic and genotypic data of the DZ twin pairs and (2) extended sib-pair variance components linkage analysis, also incorporating the phenotypic data of the MZ twin pairs. In

the standard method the phenotypic variation is decomposed into variance due to the QTL (Q), additive genetic factors (A) and unique environmental factors (E). In the extended method, the phenotypic data of the MZ twin pairs were also included, having the advantage of partitioning the phenotypic variance into effects due to the quantitative trait loci (QTL) (Q), additive genetic (A), non-additive genetic (D) or common environmental (C), and unique environmental effects (E). For the latter method, optimisation of the univariate model of the different phenotypes has been carried out. A detailed description of this optimisation procedure has been reported elsewhere,[3] and a short summary is presented in columns 2, 3 and 16 of Table 4. Our sample size is too small to conduct sex-specific linkage analyses, therefore we used a scalar model (allowing the total variances to differ between men and women) for the traits (BMI, body mass, fat body mass) that had a model with different variance components estimates for men and women as the best fitting model.[3] In the unadjusted analysis sex effects were not modeled. In these instances only simple univariate models without scalar sex effects were used. For birth weight the intra-pair correlations differ between MZ MC and MZ DC twins. Since data on chorionicity were available, extended variance component linkage analysis for birth weight was performed in three different ways: 1) using only phenotypic data of MZ DC twins, 2) using only phenotypic data of MZ MC twins, and 3) incorporating phenotypic data of both MZ MC and MZ DC twins.

**Table 2**. Anthropometric and metabolic characteristics of the MZ and DZ twins according to sex: The East Flanders Prospective Twin Survey.

| | MZ | | | DZ | | |
|---|---|---|---|---|---|---|
| Characteristic | Men | Women | *p* | Men | Women | *p* |
| *n* | 226 | 254 | | 104 | 120 | |
| Gestational age (wks)[a] | 36.8 ± 2.33 | 37.0 ± 2.59 | 0.33 | 37.4 ± 2.3 | 37.5 ± 2.2 | 0.77 |
| Birth weight (g) | 2531 ± 471 | 2451 ± 485 | 0.17 | 2694 ± 472 | 2581 ± 470 | 0.005 |
| Age (yrs)[a] | 25.2 ± 4.6 | 25.1 ± 4.6 | 0.83 | 25.1 ± 4.6 | 25.5 ± 4.6 | 0.54 |
| Body height (cm) | 178.1 ± 6.3 | 165.2 ± 6.2 | <0.0001 | 178.5 ± 6.6 | 166.7 ± 6.5 | <0.0001 |
| Body mass (kg) | 70.3 ± 9.7 | 60.5 ± 10.1 | <0.0001 | 70.1 ± 10.0 | 60.8 ± 9.6 | <0.0001 |
| BMI ($kg/m^2$)[b] | 22.0 ± 1.1 | 21.9 ± 1.2 | 0.97 | 21.9 ± 1.1 | 21.7 ± 1.1 | 0.87 |
| WHR (%) | 83.4 ± 5.5 | 73.4 ± 4.6 | <0.0001 | 82.6 ± 5.4 | 72.2 ± 4.1 | <0.0001 |
| S4SF (mm)[b] | 35.4 ± 1.5 | 56.4 ± 1.4 | <0.0001 | 33.9 ± 1.1 | 52.3 ± 1.4 | <0.0001 |
| Fat mass (kg)[b] | 11.6 ± 1.5 | 16.8 ± 1.3 | <0.0001 | 12.1 ± 1.5 | 16.6 ± 1.3 | <0.0001 |
| Lean body mass (kg) | 57.7 ± 6.5 | 43.0 ± 5.3 | <0.0001 | 56.9 ± 6.6 | 43.6 ± 5.8 | <0.0001 |
| IGFBP1 (ng/ml)[b] | 10.7 ± 1.9 | 16.7 ± 2.1 | <0.0001 | 11.9 ± 1.9 | 18.5 ± 2.2 | <0.0001 |
| Fasting insulin (pmol/l)[b] | 33.2 ± 1.5 | 37.3 ± 1.6 | 0.02 | 32.0 ± 1.6 | 40.8 ± 1.5 | 0.0001 |
| Fasting glucose (mmol/l) | 5.0 ± 0.4 | 4.5 ± 0.4 | <0.0001 | 4.8 ± 0.4 | 4.6 ± 0.4 | 0.0001 |
| Leptin (ng/ml)[b] | 1.6 ± 2.9 | 11.4 ± 2.1 | <0.0001 | 1.6 ± 2.8 | 11.6 ± 1.9 | <0.0001 |
| Total cholesterol (mmol/l) | 4.8 ± 1.0 | 5.1 ± 0.9 | 0.0008 | 4.9 ± 1.1 | 5.2 ± 0.9 | 0.03 |
| LDL-cholesterol (mmol/l) | 3.0 ± 0.9 | 2.9 ± 0.8 | 0.54 | 3.1 ± 1.0 | 2.9 ± 0.8 | 0.06 |
| HDL-cholesterol (mmol/l) | 1.3 ± 0.3 | 1.8 ± 0.4 | <0.0001 | 1.4 ± 0.4 | 1.9 ± 0.4 | <0.0001 |
| Triglycerides (mmol/l)[b] | 0.9 ± 1.5 | 0.9 ± 1.5 | 0.32 | 0.9 ± 1.5 | 0.9 ± 1.3 | 0.85 |
| NEFA (mmol/l) | 0.5 ± 0.2 | 0.7 ± 0.2 | <0.0001 | 0.5 ± 0.2 | 0.7 ± 0.2 | <0.0001 |

Data are expressed as mean ± SD. [a]*p*-value calculated using standard linear regression, because convergence criteria could not be met in a random intercept model. [b]Geometric mean ± SD.

Estimates of the variance component associated with the QTL were obtained using the ĥ-approach, in which the covariance due to the marker or QTL for a sib-pair is modeled as a function of the proportion of alleles shared identical by descent (IBD). Since parental data were not available, the probabilities of sharing 0, 1 or 2 alleles IBD were estimated in MERLIN[25] using allele frequencies based on one randomly selected co-twin per pair (using genotypes of MZ and DZ twin pairs). The effect of the QTL was evaluated by comparing a full model, in which the genetic variance caused by Q for a given phenotype was free, with a restricted model, in which the effect of Q was equal to zero. Logarithm of the odds (LOD) scores, computed as the difference in –2log likelihood divided by 4.6, were used to evaluate the QTL effect. Because we used a candidate gene approach, the multiple testing problem associated with a genome-wide search does not apply.[27] Suggestive evidence for linkage was defined by LOD>1 and significant evidence for linkage by LOD>3.

## 3.4 RESULTS

### *3.4.1 General statistical analysis*

In Table 2 anthropometric and metabolic characteristics of the MZ and DZ twins are expressed as mean ± SD according to sex. In both MZ and DZ twins, men had a higher body height, body mass, WHR, lean body mass and fasting glucose levels, but lower sum of four skinfolds (S4SF), fat mass, IGFBP1, fasting insulin, leptin, total cholesterol, HDL-cholesterol and NEFA levels than women ($p$<0.05). In DZ twins only, men had a higher weight at birth ($p$<0.05).

### *3.4.2 Linkage Analysis*

Microsatellite markers D7S2427 (*GCK*) and D7S478 (*IGFBP1*) deviated from the assumptions of Hardy-Weinberg equilibrium ($p$<0.05) and were excluded from further linkage analyses.
Results of the univariate single-point variance component linkage analyses performed using the standard and the extended method, unadjusted and adjusted for significant covariates, are listed in Table 3 and Table 4. After adjustment, the D11S902 (*ABCC8*) marker showed suggestive linkage with WHR, and with HDL-cholesterol levels using the extended method only. Before adjustment, linkage with HDL-cholesterol levels was non-significant. The D3S1602 (*ADIPOQ*) marker showed in the adjusted analysis suggestive linkage with body mass and HDL-cholesterol levels using both methods, and with triglycerides levels using the extended method only. Before controlling for covariates linkage with triglycerides levels was non-significant and suggestive linkage with body height and lean body mass was observed. The downstream marker D3S3686 showed also suggestive linkage with triglycerides levels using both methods, while before adjustment this linkage was non-significant and linkage with HDL-cholesterol levels seemed suggestive.
After controlling for covariates, the D7S2506 marker (*IGFBP1*) showed suggestive linkage with leptin levels using both methods, with fat mass using the standard method only and with fasting insulin using the extended method only. Before adjusting, this marker showed also suggestive linkage with the sum of four skinfolds, and with fat mass it showed significant linkage.

**Table 3**. Results (LOD scores) of univariate single-point variance components linkage analyses unadjusted for significant covariates performed using the standard (S) and the extended (E) method.

| | | | *ABCC8* | *ADIPOQ* | | *GCK* | *IGF1* | *IGFBP1* | *INSR* | *LEP* | *LEPR* | | *PPARγ* | *RETN* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Trait | Method | Model | D11S902 | D3S1602 | D3S3686 | GCK | IGF1 | D7S2506 | D19S1034 | D7S530 | D1S2638 | D1S198 | D3S1263 | D19S413 |
| Birth weight | S | | 0.00 | 0.00 | 0.15 | 0.00 | 0.00 | 0.21 | 0.11 | **1.13** | 0.00 | 0.07 | 0.13 | 0.52 |
| | $E_{MZDConly}$ | ACE | 0.00 | 0.03 | 0.61 | 0.00 | 0.00 | 0.19 | 0.01 | **1.12** | 0.00 | 0.00 | 0.27 | 0.39 |
| | $E_{MZMConly}$ | ACE | 0.00 | 0.02 | 0.31 | 0.00 | 0.00 | 0.11 | 0.01 | 0.41 | 0.00 | 0.01 | 0.13 | 0.30 |
| | E | ACE | 0.00 | 0.03 | 0.56 | 0.00 | 0.00 | 0.18 | 0.01 | 0.78 | 0.00 | 0.00 | 0.24 | 0.43 |
| Body height | S | | 0.46 | **1.33** | 0.67 | 0.03 | 0.00 | 0.00 | 0.00 | 0.06 | 0.00 | 0.16 | 0.03 | 0.00 |
| | E | AE | 0.51 | **1.17** | 0.60 | 0.06 | 0.00 | 0.00 | 0.00 | 0.09 | 0.00 | 0.10 | 0.02 | 0.00 |
| Body mass | S | | 0.11 | **2.07** | 0.20 | 0.00 | 0.00 | 0.00 | 0.00 | 0.08 | 0.53 | 0.00 | 0.50 | 0.00 |
| | E | AE | 0.15 | **1.98** | 0.16 | 0.00 | 0.00 | 0.00 | 0.00 | 0.04 | 0.60 | 0.00 | 0.32 | 0.00 |
| BMI | S | | 0.00 | 0.47 | 0.07 | 0.00 | 0.00 | 0.49 | 0.00 | 0.11 | 0.11 | 0.00 | 0.41 | 0.00 |
| | E | AE | 0.00 | 0.56 | 0.08 | 0.00 | 0.00 | 0.76 | 0.00 | 0.20 | 0.00 | 0.00 | 0.62 | 0.00 |
| WHR | S | | **1.02** | 0.21 | 0.19 | 0.28 | 0.00 | 0.00 | 0.00 | 0.32 | 0.10 | 0.00 | 0.00 | 0.00 |
| | E | AE | **1.39** | 0.05 | 0.09 | 0.23 | 0.00 | 0.00 | 0.00 | 0.29 | 0.34 | 0.00 | 0.00 | 0.00 |
| S4SF | S | | 0.00 | 0.58 | 0.29 | 0.00 | 0.00 | **2.03** | 0.00 | 0.00 | 0.00 | 0.01 | 0.00 | 0.00 |
| | E | AE | 0.00 | 0.53 | 0.29 | 0.00 | 0.00 | **2.19** | 0.00 | 0.00 | 0.00 | 0.04 | 0.00 | 0.00 |
| Fat mass | S | | 0.00 | 0.88 | 0.27 | 0.00 | 0.00 | **3.64** | 0.00 | 0.00 | 0.44 | 0.00 | 0.54 | 0.00 |
| | E | AE | 0.00 | 1.00 | 0.24 | 0.00 | 0.00 | **3.53** | 0.00 | 0.00 | 0.22 | 0.00 | 0.53 | 0.00 |
| Lean body mass | S | | 0.56 | **1.56** | 0.70 | 0.00 | 0.00 | 0.00 | 0.00 | 0.06 | 0.45 | 0.21 | 0.05 | 0.00 |
| | E | AE | 0.61 | **1.34** | 0.66 | 0.01 | 0.00 | 0.00 | 0.00 | 0.06 | 0.49 | 0.17 | 0.03 | 0.00 |
| IGFBP1 | S | | 0.00 | 0.03 | 0.10 | 0.02 | 0.25 | 0.11 | 0.00 | 0.00 | 1.00 | 0.00 | 0.01 | 0.00 |
| | E | DE | 0.00 | 0.03 | 0.13 | 0.06 | 0.25 | 0.12 | 0.00 | 0.04 | **1.12** | 0.00 | 0.03 | 0.04 |
| Fasting insulin | S | | 0.30 | 0.00 | 0.00 | 0.00 | 0.00 | 0.97 | 0.00 | **1.09** | 0.59 | 0.09 | 0.00 | 0.00 |
| | E | DE | 0.60 | 0.02 | 0.04 | 0.19 | 0.22 | **1.28** | 0.03 | **1.43** | 0.86 | 0.35 | 0.00 | 0.01 |
| Fasting glucose | S | | 0.00 | 0.00 | 0.00 | 0.11 | 0.00 | 0.17 | 0.92 | 0.30 | 0.00 | 0.00 | 0.00 | 0.48 |
| | E | AE | 0.00 | 0.00 | 0.00 | 0.12 | 0.00 | 0.16 | **1.04** | 0.32 | 0.00 | 0.00 | 0.00 | 0.42 |
| Leptin | S | | 0.00 | 0.06 | 0.00 | 0.01 | 0.00 | 0.88 | 0.01 | 0.07 | 0.07 | 0.39 | 0.05 | 0.00 |
| | E | AE | 0.00 | 0.03 | 0.00 | 0.04 | 0.00 | **1.21** | 0.03 | 0.04 | 0.06 | 0.49 | 0.05 | 0.00 |
| Total cholesterol | S | | 0.05 | 0.03 | 0.00 | 0.00 | 0.05 | 0.01 | 0.18 | 0.00 | 0.04 | 0.07 | 0.00 | 0.01 |
| | E | ACE | 0.03 | 0.03 | 0.00 | 0.00 | 0.18 | 0.00 | 0.14 | 0.00 | 0.13 | 0.11 | 0.00 | 0.04 |
| LDL-cholesterol | S | | 0.23 | 0.00 | 0.00 | 0.00 | 0.19 | 0.00 | 0.03 | 0.00 | 0.00 | 0.02 | 0.00 | 0.00 |
| | E | ACE | 0.37 | 0.00 | 0.00 | 0.00 | 0.70 | 0.00 | 0.00 | 0.00 | 0.19 | 0.14 | 0.00 | 0.00 |
| HDL-cholesterol | S | | 0.84 | **2.95** | **2.49** | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.19 |
| | E | AE | 0.92 | **2.96** | **2.42** | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.22 |
| Triglycerides | S | | 0.00 | 0.79 | 0.60 | 0.49 | 0.00 | 0.34 | 0.04 | 0.18 | 0.01 | 0.00 | 0.56 | 0.00 |
| | E | AE | 0.00 | 0.84 | 0.63 | 0.41 | 0.00 | 0.37 | 0.04 | 0.19 | 0.00 | 0.00 | 0.57 | 0.00 |
| NEFA | S | | 0.68 | 0.06 | 0.16 | 0.00 | 0.44 | 0.00 | 0.00 | 0.00 | 0.21 | 0.02 | 0.00 | 0.00 |
| | E | AE | 0.63 | 0.04 | 0.13 | 0.00 | 0.38 | 0.00 | 0.00 | 0.00 | 0.20 | 0.02 | 0.00 | 0.00 |

**Bold and underlined numbers**: LOD>1 and LOD<3 suggestive evidence for linkage; LOD>3 significant evidence for linkage. E = extended method, $E_{MZDConly}$ = only phenotypic data of MZ DC twins incorporated, $E_{MZMConly}$ = only phenotypic data of MZ MC twins incorporated, S = standard method.

**Table 4**. Results (LOD scores) of univariate single-point variance components linkage analyses adjusted for significant covariates performed using the standard (S) and the extended (E) method.

| | | | *ABCC8* | *ADIPOQ* | | *GCK* | *IGF1* | *IGFBP1* | *INSR* | *LEP* | *LEPR* | | *PPARγ* | *RETN* | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Trait | Method | Model | D11S902 | D3S1602 | D3S3686 | GCK | IGF1 | D7S2506 | D19S1034 | D7S530 | D1S2638 | D1S198 | D3S1263 | D19S413 | Covariates |
| Birth weight | S | | 0.00 | 0.02 | 0.20 | 0.04 | 0.00 | 0.07 | 0.03 | **1.02** | 0.00 | 0.00 | 0.32 | 0.61 | Sex, gestational |
| | $E_{MZDConly}$ | ACE | 0.00 | 0.02 | 0.23 | 0.03 | 0.00 | 0.06 | 0.03 | 0.97 | 0.00 | 0.00 | 0.37 | 0.57 | age |
| | $E_{MZMConly}$ | ACE | 0.00 | 0.02 | 0.13 | 0.00 | 0.00 | 0.07 | 0.02 | 0.26 | 0.00 | 0.00 | 0.16 | 0.35 | |
| | E | ACE | 0.00 | 0.02 | 0.25 | 0.01 | 0.00 | 0.08 | 0.03 | 0.63 | 0.00 | 0.00 | 0.31 | 0.58 | |
| Body height | S | | 0.00 | 0.95 | 0.14 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.06 | 0.67 | 0.00 | Sex, age |
| | E | AE | 0.00 | 0.86 | 0.11 | 0.00 | 0.00 | 0.01 | 0.00 | 0.00 | 0.00 | 0.07 | 0.66 | 0.00 | |
| Body mass | S | | 0.00 | **1.59** | 0.00 | 0.00 | 0.00 | 0.41 | 0.00 | 0.02 | 0.56 | 0.00 | 0.52 | 0.00 | Sex, age, height |
| | E | AE[a] | 0.00 | **1.50** | 0.01 | 0.00 | 0.00 | 0.29 | 0.00 | 0.05 | 0.24 | 0.00 | 0.35 | 0.00 | |
| BMI | S | | 0.00 | 0.47 | 0.05 | 0.00 | 0.00 | 0.50 | 0.00 | 0.16 | 0.12 | 0.00 | 0.41 | 0.00 | Age |
| | E | AE[a] | 0.00 | 0.50 | 0.09 | 0.00 | 0.00 | 0.47 | 0.00 | 0.42 | 0.00 | 0.00 | 0.33 | 0.00 | |
| WHR | S | | **1.02** | 0.01 | 0.00 | 0.37 | 0.00 | 0.00 | 0.00 | 0.59 | 0.02 | 0.00 | 0.03 | 0.00 | Sex, age |
| | E | AE[a] | **1.09** | 0.00 | 0.00 | 0.48 | 0.00 | 0.00 | 0.00 | 0.68 | 0.09 | 0.00 | 0.01 | 0.00 | |
| S4SF | S | | 0.00 | 0.53 | 0.29 | 0.00 | 0.00 | 0.74 | 0.00 | 0.00 | 0.03 | 0.00 | 0.00 | 0.00 | Sex, age |
| | E | AE[a] | 0.00 | 0.71 | 0.36 | 0.00 | 0.00 | 0.66 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | |
| Fat mass | S | | 0.00 | 0.67 | 0.21 | 0.01 | 0.02 | **1.20** | 0.00 | 0.13 | 0.18 | 0.00 | 0.41 | 0.00 | Sex, age |
| | E | AE[a] | 0.00 | 0.75 | 0.20 | 0.05 | 0.00 | 0.85 | 0.00 | 0.23 | 0.12 | 0.00 | 0.46 | 0.00 | |
| Lean body mass | S | | 0.03 | 0.97 | 0.05 | 0.00 | 0.00 | 0.02 | 0.00 | 0.04 | 0.16 | 0.00 | 0.02 | 0.00 | Sex, age, height |
| | E | AE[a] | 0.00 | 0.92 | 0.00 | 0.00 | 0.00 | 0.14 | 0.00 | 0.01 | 0.27 | 0.00 | 0.09 | 0.00 | |
| IGFBP1 | S | | 0.00 | 0.02 | 0.26 | 0.09 | 0.08 | 0.20 | 0.00 | 0.00 | 0.56 | 0.00 | 0.01 | 0.00 | Sex, age, BMI |
| | E | DE[a] | 0.00 | 0.01 | 0.40 | 0.09 | 0.07 | 0.13 | 0.00 | 0.00 | 0.55 | 0.00 | 0.04 | 0.03 | |
| Fasting insulin | S | | 0.11 | 0.05 | 0.05 | 0.00 | 0.00 | 0.76 | 0.00 | 0.29 | 0.46 | 0.07 | 0.00 | 0.00 | Age, S4SF |
| | E | DE | 0.31 | 0.18 | 0.28 | 0.11 | 0.16 | **1.13** | 0.07 | 0.66 | 0.70 | 0.31 | 0.00 | 0.00 | |
| Fasting glucose | S | | 0.00 | 0.00 | 0.00 | 0.19 | 0.00 | 0.17 | 0.91 | 0.10 | 0.00 | 0.00 | 0.05 | 0.23 | Sex, BMI |
| | E | AE[a] | 0.00 | 0.00 | 0.00 | 0.28 | 0.00 | 0.08 | 0.70 | 0.16 | 0.00 | 0.00 | 0.11 | 0.09 | |
| Leptin | S | | 0.01 | 0.37 | 0.15 | 0.02 | 0.34 | **1.54** | 0.00 | 0.00 | 0.18 | 0.00 | 0.00 | 0.00 | Sex, age, S4SF |
| | E | AE[a] | 0.00 | 0.36 | 0.22 | 0.32 | 0.12 | **1.42** | 0.00 | 0.00 | 0.21 | 0.00 | 0.00 | 0.00 | |
| Total cholesterol | S | | 0.02 | 0.00 | 0.00 | 0.00 | 0.28 | 0.00 | 0.09 | 0.00 | 0.12 | 0.30 | 0.00 | 0.11 | Age, S4SF |
| | E | AE | 0.03 | 0.00 | 0.00 | 0.00 | 0.04 | 0.00 | 0.15 | 0.00 | 0.00 | 0.12 | 0.00 | 0.04 | |
| LDL-cholesterol | S | | 0.07 | 0.00 | 0.00 | 0.04 | 0.43 | 0.00 | 0.05 | 0.00 | 0.14 | 0.22 | 0.00 | 0.00 | Sex, age, S4SF |
| | E | AE | 0.02 | 0.00 | 0.00 | 0.06 | 0.05 | 0.00 | 0.10 | 0.00 | 0.00 | 0.06 | 0.00 | 0.00 | |
| HDL-cholesterol | S | | 0.93 | **1.83** | 0.57 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.48 | Sex, S4SF |
| | E | AE[a] | **1.09** | **1.88** | 0.70 | 0.00 | 0.01 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.47 | |
| Triglycerides | S | | 0.00 | 1.00 | **1.09** | 0.73 | 0.00 | 0.45 | 0.02 | 0.00 | 0.00 | 0.00 | 0.28 | 0.00 | Sex, S4SF |
| | E | AE | 0.00 | **1.15** | **1.42** | 0.58 | 0.00 | 0.64 | 0.04 | 0.00 | 0.00 | 0.00 | 0.17 | 0.00 | |
| NEFA | S | | 0.28 | 0.16 | 0.15 | 0.00 | 0.27 | 0.00 | 0.01 | 0.00 | 0.19 | 0.02 | 0.00 | 0.00 | Sex, S4SF |
| | E | AE[a] | 0.21 | 0.05 | 0.13 | 0.00 | 0.27 | 0.00 | 0.00 | 0.00 | 0.18 | 0.01 | 0.00 | 0.00 | |

**Bold and underlined numbers**: LOD>1 suggestive evidence for linkage. E = extended method, $E_{MZDConly}$ = only phenotypic data of MZ DC twins incorporated, $E_{MZMConly}$ = only phenotypic data of MZ MC twins incorporated, S = standard method. [a]Scalar effect.

After adjustment, the D7S530 (*LEP*) marker showed suggestive linkage with birth weight using the standard method only. Before controlling for covariates, also suggestive linkage with fasting insulin levels was observed.
The D1S2638 (*LEPR*) and the D19S1034 (*INSR*) marker showed in the unadjusted analysis suggestive linkage with IGFBP1 and with fasting glucose levels, respectively. However, after adjustment these linkages became non-significant.

## 3.5 DISCUSSION

In the current study, a series of anthropometric and metabolic characteristics were measured in 240 MZ and 112 DZ twin pairs recruited from the EFPTS. Fourteen microsatellite markers located within 10 candidate gene regions were genotyped and univariate single point variance components linkage analyses were performed. The initial DZ twin sample comprises 138 twin pairs, of which 26 DZ twin pairs did not provide DNA. As differences in mean age (25.4 *vs* 25.7), weight (65.1 *vs* 65.8) and height (172.1 *vs* 172.1) are negligible between the two samples, the genotyped sample is still representative for the initial DZ twin sample. Two microsatellite markers, one located near *GCK* (D7S2427) and the other near *IGFBP1* (D7S478), deviated from the assumptions of Hardy-Weinberg equilibrium (HWE). Deviation from HWE can occur due to genotyping errors, inbreeding, genetic drift, selection and population stratification. Because the last four causes have impact on the whole genome and in the current study only 2 of the 14 markers deviated from HWE, the most likely reason for deviation from HWE is genotyping errors, presumably systematic mistyping of heterozygotes as homozygotes.[28] To avoid false positive linkage results, these markers were excluded from further linkage analyses.
Linkage analyses were carried out unadjusted as well as adjusted for significant covariates, resulting in considerable differences between the linkage results (Table 3 and Table 4). In many occasions, unadjusted LOD scores were larger than 1 and gave therefore suggestive evidence for linkage, but after adjusting for covariates LOD scores were non-significant. The D7S2506 marker (*IGFBP1*) for example showed suggestive linkage with the sum of four skinfolds ($LOD_S$=2.03; $LOD_E$=2.19) in the unadjusted analysis, but after adjusting for sex and age the LOD scores were non-significant ($LOD_S$=0.74; $LOD_E$=0.66). The opposite also occurred, where LOD scores were smaller than 1 in the unadjusted analysis, but gave suggestive evidence for linkage after controlling for significant covariates. For example, linkage of the D3S3686 marker (*ADIPOQ*) with triglycerides levels was not significant in the unadjusted analysis ($LOD_S$=0.60; $LOD_E$=0.63), but after adjusting for covariates linkage was suggestive ($LOD_S$=1.09; $LOD_E$=1.42). These data indicate that controlling for covariates has great impact on the final linkage results and without adjustment false positive and false negative outcomes are eminent and may lead to futile follow up studies.

In the present study DNA was only available of 112 DZ twin pairs, and consequently our power to detect QTLs is rather low. In addition, we recognise that due to the large number of statistical tests performed some of the results reported here may represent false-positive findings and therefore our results must be interpreted cautiously. Nevertheless, 5 out of 12 microsatellite markers, located in 4 candidate gene regions (*ABCC8, ADIPOQ*, *IGFBP1* and *LEP*), showed after adjusting for covariates suggestive linkage with several anthropometric

and metabolic characteristics. The D11S902 marker close to the *ABCC8* gene showed suggestive linkage with WHR and HDL-cholesterol levels. 4.5 kb downstream of *ABCC8,* the *KCNJ11* gene encoding the other subunit of the $K_{ATP}$ channel (Kir6.2) is located. This gene contains the E23K single nucleotide polymorphism (SNP), which is a well-established genetic risk factor for T2D and which is in strong linkage disequilibrium with the coding SNP A1369S (rs757110) in exon 33 of *ABCC8*.[29,30] However, the E23K SNP has been genotyped in our twin sample and association analysis revealed that this SNP did not explain the observed linkage signal (data not shown). This indicates that besides the *KCNJ11* E23K SNP, other genetic variants predisposing to T2D are located in the *ABCC8* gene region. For example, SNPs in the promoter of *ABCC8* that have been associated with an increased T2D risk in a Finnish sample.[31] Nevertheless, the linkage signal might also be the consequence of rare population specific variants.

The D3S1602 marker, upstream the *ADIPOQ* gene, showed evidence for suggestive linkage with body mass, HDL-cholesterol and triglycerides levels. The D3S3686 marker, located downstream of *ADIPOQ*, also showed linkage with triglycerides levels, suggesting that a genetic variant close to the markers influences this particular trait. Several genome wide scans reported evidence for linkage of the 3q27 locus with anthropometric and metabolic characteristics.[32-34] Moreover, a genome-wide linkage scan of plasma adiponectin levels in the Amish Family Diabetes Study reported the highest LOD score between markers D3S1602 and D3S1580, which also flank the *ADIPOQ* gene, as in our study.[33] Several polymorphisms in *ADIPOQ* have already been associated with circulating adiponectin levels, obesity, insulin resistance and cardiovascular disease risk.[33,35-41] However, more T2D candidate genes are located in the *ADIPOQ* gene region and accordingly the observed linkage signals might also be the result of genetic variation in one of these other genes. For example, the somatostatin (*SST*) gene which is in the close vicinity of the D3S3686 marker, and the diacylglycerol kinase gamma (*DGKG*) gene or the insulin-like growth factor 2 mRNA binding protein 2 (*IGF2BP2*) gene that are located near the D3S1602 marker.

The D7S2506 marker located in the *IGFBP1* gene region showed suggestive linkage with fat mass, fasting insulin and leptin levels. Until now, just one genome wide linkage scan reported suggestive linkage of this region (D7S1818; LOD=2.20) with trends in BMI from childhood to adulthood.[42] Probably as a consequence, only few association studies with *IGFBP1* have been carried out. Ukkola et al.[43] reported association with an intronic SNP and overfeeding induced changes in abdominal visceral fat, OGTT (oral glucose tolerance test) insulin area and total cholesterol levels in twins, and a coding SNP (A4403G) has been associated with impaired renal function in T2D.[44] Although *IGFBP1* has not been studied very intensively, our linkage results and the strong phenotypic correlation of IGFBP1 levels with other metabolic risk factors suggest an interesting role of the *IGFBP1* gene in the development of the metabolic syndrome.[15,45] The fact that we did not observe linkage between the D7S2506 marker and IGFBP1 levels is remarkable. However, in a genome wide linkage analysis using expression phenotypes, more *trans*-acting QTLs than *cis*-acting QTLs (mapped within 5Mb of the target gene) were observed.[46] This indicates that even gene expression levels are complex traits and transcription regulators are not by definition closely located to the gene. In addition, it should be noted that the *IGFBP1* gene is located closely to the *IGFBP3* gene, and therefore *IGFBP3* could also be responsible for the observed linkage signal.

The D7S530 marker located near the *LEP* gene (7q32.2) showed suggestive linkage with birth weight. Linkage of the 7q32.3 locus with birth weight has, as far as we know, not been reported previously. However, a growth-promoting role for leptin during fetal development has been suggested before,[47] since umbilical cord leptin levels are positively correlated with birth weight.[48] Moreover, we recently showed that two polymorphisms in the leptin receptor (*LEPR*) gene predispose to a higher weight at birth.[49] Interestingly, in the present study the two microsatellite markers located near the *LEPR* gene showed no linkage with birth weight. To detect a QTL for a complex trait like birth weight, which is controlled by many loci with small effects, association studies are more powerful than linkage studies. Hence, linkage signals are probably not the result of one polymorphism, but represent a joint effect of several (common and rare) variants.
In the literature the *GCK*, *IGF1*, *INSR*, *LEP, LEPR*, *PPARγ* and *RETN* gene regions have been associated with T2D or traits related to this disease.[5] In the current study we did not detect linkage between traits related to T2D and markers located within these gene regions. This does not imply that these regions do not contribute to the development of T2D in the current sample, but the studied sample or the contribution of these loci to the traits may be too small to detect them in this linkage analysis.

We compared two methods when performing univariate single point variance components linkage analysis. The standard method comprised only phenotypic and genotypic data of the DZ twin pairs and is therefore equal to the sib-pair based variance component linkage analysis implemented in MERLIN.[25] In the extended method, which is implemented in Mx,[24] the phenotypic data of the MZ twin pairs were also incorporated and model fitting was carried out to obtain the best-fitting model. This results in a more accurate estimation of the variance components compared to the standard method. Despite the differences between the two methods used, they gave fairly similar linkage results. However, for fasting insulin, of which the variance is partly explained by non-additive genetic factors (DE model), LOD scores were remarkably higher using the extended method compared to the standard method (see Table 3 and Table 4). This might be the result of the low DZ twin correlations, because when non-additive genetic factors are present DZ twin correlations are less than half of the MZ twin correlations. In the standard method the size of the genetic component is estimated based on data of DZ twins only, and we observed that the size of the genetic component in the standard method was lower than in the extended method. Our data may suggest that the underestimation of the genetic effect size in the standard method also results in an underestimation of the QTL effect. This indicates, that when non-additive genetic factors are present the extended method is more powerful than the standard method. However, non-additive genetic factors also explain part of the variance of IGFBP1 levels and for this trait LOD scores were not consistently higher using the extended method. Moreover, our sample size and the number of microsatellite markers tested are limited, and therefore these result should be interpreted carefully.
We also performed linkage analyses with birth weight. Because MZ MC twins suffer a more adverse intrauterine environment than MZ DC twins, intra-pair correlations for birth weight are significantly lower for MZ MC compared to MZ DC twins. Since the EFPTS has data on chorionicity available, we were able to compare four different methods for linkage analysis with birth weight: 1) the standard method, 2) the extended method incorporating only phenotypic data of MZ DC twins, 3) the extended method incorporating only phenotypic data

of MZ MC twins, and 4) the extended method in which phenotypic data of both MZ MC and MZ DC were incorporated (see Table 3 en 4). In general, the four methods gave similar results, except for the D7S530 marker that showed suggestive linkage using the standard method ($LOD_{Sunadjusted}$=1.13, $LOD_{Sadjusted}$=1.02) and the extended method in which only the phenotypic data of the MZ DC twins were incorporated ($LOD_{E(MZDConly)unadjusted}$=1.12, $LOD_{E(MZDConly)adjusted}$=0.97). However, LOD scores for the D7S530 marker were considerably lower in the methods incorporating phenotypic data of the MZ MC twins ($LOD_{E(MZMConly)unadjusted}$=0.41, $LOD_{E(MZMConly)adjusted}$=0.26; $LOD_{Eunadjusted}$=0.78, $LOD_{Eadjusted}$=0.63). Probably due to the low covariance of the MZ MC twins, the size of the genetic effect and consequently the QTL effect was underestimated. This suggests that including phenotypic data of MZ MC twins results in a reduced power to detect a QTL for birth weight using variance component linkage analyses. In addition, these results indicate that when chorionicity is an important confounder and data on chorionicity is unavailable, variance component linkage analysis should be performed using data of DZ twins only.
Although the sample size and the number of microsatellite markers tested in the present study are limited, our data suggest that genetic variations located in the *ABCC8, ADIPOQ, IGFBP1* and *LEP* gene regions influence anthropometric and metabolic characteristics in the East Flanders twin sample. In addition, our data show that carrying out sib-pair based variance components linkage analyses by either using advanced modelling in Mx or using the straightforward standard method implemented in the MERLIN package yields similar results, at least when chorionicity is not an important confounder.

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# CHAPTER 4

# THE EFFECT OF PREVIOUSLY REPORTED GENETIC RISK FACTORS ON BIRTH WEIGHT AND TYPE 2 DIABETES RELATED TRAITS IN YOUNG ADULT TWINS

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*Submitted*

## 4.1 ABSTRACT

Despite the numerous association studies performed, the role of many SNPs in the etiology of T2D are still unclear and the mechanisms underlying reported associations remain largely unknown. We therefore examined the effect of eleven SNPs, that have previously been associated with T2D and/or disease related phenotypes, on birth weight and adult metabolic risk factors measured in young adults. Eleven SNPs in eleven genes were genotyped in 197 monozygotic, 108 dizygotic and 18 single twins (286 men and 342 women, mean age 25) recruited from the East Flanders Prospective Twin Survey. In the complete sample, P12A (*PPARγ*) showed association with birth weight and WHR ($p$=0.04 and $p$=0.02), -174G>C (*IL6*) with HDL-cholesterol ($p$=0.008), and -243A>G (*GAD2*) with fasting insulin levels ($p$=0.01). In men only, -174G>C (*IL6*) was associated with NEFA ($p$=0.005), -243A>G (*GAD2*) with lean body mass and fasting insulin ($p$=0.004 and $p$=0.004), K121Q (*ENPP1*) with NEFA ($p$=0.02) and P129T (*FAAH*) with birth weight ($p$=0.02). In women only, -174G>C (*IL6*) was associated with the sum of four skinfolds and triglycerides ($p$=0.002 and $p$=0.04), R72T (*PYY*) with fasting glucose ($p$=0.003), K121Q (*ENPP1*) with HDL-cholesterol ($p$=0.05), and -866G>A (*UCP2*) with LDL-cholesterol levels ($p$=0.01). No associations were observed for E23K (*KCNJ11*), 1166A>C (*AGTR1*), 6235T>C (*CYP1A1*) and G1057D (*IRS2*). In these young adults, seven out of eleven previously reported genetic risk factors of T2D were associated with one or more metabolic risk factors. Most of those were sex-specific.

## 4.2 INTRODUCTION

Abundant nutrient supply combined with a sedentary lifestyle has resulted in a dramatic rise of the worldwide incidence of type 2 diabetes (T2D), a disease currently considered as one of the major causes of premature illness and death.[1] In addition to the key role of lifestyle factors, genetic factors also play an important role in the etiology of T2D as shown by family and twin studies.[1,2] A better understanding of these genetic factors could improve the identification of novel therapeutic targets and therefore much effort has been put into the elucidation of the genetics of T2D.[3]

To uncover regions harbouring candidate genes for T2D, numerous genome wide linkage scans have been performed.[3] Although linkage analysis has been powerful in the identification of rare high-risk disease alleles, it has been less successful in identifying common disease variants with small effects.[4] The transcription factor 7-like 2 (*TCF7L2*) gene is thus far the only T2D predisposing gene identified by linkage analysis, which has thereafter consistently been replicated in other populations.[5,6]

For the detection of common disease alleles with modest disease risk, association studies are more successful.[4] Because of the high costs of genome-wide association studies, most association studies carried out are from the candidate-gene type; where the relation between T2D and single nucleotide polymorphisms (SNPs) in either positional (within genomic regions revealed by linkage studies) but mostly functional (related to disease pathology) candidates genes have been studied.[3] Some SNPs have been reproducibly associated with T2D in multiple studies, including the E23K SNP of the *KCNJ11* gene encoding the Kir6.2 subunit of the ATP-sensitive potassium channel and the P12A SNP of the peroxisome proliferator activated receptor-γ (*PPARγ*).[6-8]

However, the association between T2D and SNPs in other candidate gene regions is less well established. This is mainly due to the lack of success in replicating reported associations in other populations. Failures in replication may be caused by the threshold conditions used to classify cases and controls, and the variable age of onset of the disease; apparently unaffected individuals may become diabetic later in life and therefore differential misclassification of controls may have occurred.[3] Considering the heterogeneity of the disease and the knowledge that T2D in general is the result of a prolonged clustering of several metabolic abnormalities (obesity, glucose intolerance/insulin resistance, dyslipidaemia), studying these metabolic risk factors in stead of defining cases and controls will likely give more consistent results. Genes that influence traits at a lower level of the physiological hierarchy might be easier to map because the phenotypes they influence are closer to the "genetic substrate" that determines them. Hence, the total number of genes influencing these intermediate traits is likely to be less than the total number of genes involved in the disease.[9] Furthermore, this approach will provide immediate information about the specific metabolic pathway in which the genetic variant is active.
In this study we examined whether SNPs located in known or suspected T2D candidate genes are associated with birth weight and adult metabolic risk factors in a young population-based twin sample recruited from the East-Flanders Prospective Twin Survey (EFPTS).[2] SNPs were selected in genes that are involved in insulin secretion (potassium inwardly-rectifying channel, subfamily J, member 11, *KCNJ11*), insulin signalling (ectoenzyme nucleotide pyrophosphate phosphodiesterase 1, *ENPP1*; insulin receptor substrate 2, *IRS2*), adipocyte functioning (*PPARγ*), inflammatory processes (interleukin 6, *IL6*), detoxification (cytochrome P450 1A1, *CYP1A1*), feeding behaviour (peptide YY, *PYY;* fatty acid amide hydrolase, *FAAH*; glutamate decarboxylase 2, *GAD2*), intra-mitochondrial energy transport (uncoupling protein 2, *UCP2*) and physiological processes of the cardiovascular system (angiotensin II type 1 receptor, *AGTR1*). All selected SNPs have previously been associated with T2D and/or with metabolic characteristics related to the disease in other populations, and are either coding variants or located in regions that might be of importance in the regulation of gene expression (Table 1). Two of the SNPs examined in this study (*KCNJ11* E23K and *PPARγ* P12A) have recently been replicated in a large genome wide association study;[6] the role of the other SNPs in the etiology of T2D is still inconclusive (see supplementary material II Table S5-S15).

## 4.3 MATERIALS AND METHODS

### *4.3.1 Participants*

The EFPTS is a population-based twin register, which started in 1964 and has been recording all multiple births in the Belgian Province of East Flanders until the present. A detailed description of the twin sample has been given elsewhere.[2,10] Phenotypic data and DNA were available of 628 individuals, including 197 monozygotic (MZ) and 108 dizygotic (DZ) complete twin pairs and 18 single twins (2 MZ/16 DZ). For the analysis of lipid and carbohydrate parameters, subjects taking drugs with potential effects on the lipid or carbohydrate metabolism were excluded (n=11). The Ethics Committee of the Faculty of Medicine of the Katholieke Universiteit Leuven approved the project and all participants gave informed consent.

**Table 1.** Single nucleotide polymorphisms (SNPs) genotyped, primer sequences and PCR conditions.

| *Gene* | RefSNPnr | Location | DNA change | Protein change | Primer sequence (5'→3') | PSQ primer (5'→3') | Restriction enzyme | Size (bp) | $MgCl_2$ (mM) | dNTP (nM) | C | T (°C) | GR (%) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *PPARγ* | rs1801282 | coding | 34C>G | P12A | **F**:TCAAGCCCAGTCCTTTCTGT<br>**R**:Biotin-CCCAATAGCCGTATCTGGAA | **F**:GGGAGATTCTCCTATTGAC | - | 296 | 1.5 | 200[b] | 50 | 55 | 96.5 |
| *IL6* | rs1800795 | promoter | -174G>C | - | **F**:Biotin-TCGTGCATGACTTCAGCTTT<br>**R**:TCATGGGAAAATCCCACATT | **R**:TGTGACGTCCTTTAGCA | - | 272 | 1.5 | 200[b] | 50 | 55 | 93.7 |
| *GAD2* | rs2236418 | promoter | -243A>G | - | **F**:CTTTCCCTCAAATGCTCTGG<br>**R**:AGAAAACGTGCGTGTCTGTG | - | Dra I | 168 | 1.5 | 330[c] | 35 | 55 | 96.5 |
| *PYY* | rs1058046 | coding | 214G>C | R72T | **F**:Biotin-CCAGATCTGACCACGCTCTT<br>**R**:AGTGATGTTGCCAGGGTAGG | **R**:CGTTTTGGAAAGAAGC | - | 187 | 1.5[a] | 330[c] | 50 | 55 | 97.2 |
| *ENPP1*[11] | rs1044498 | coding | 361A>C | K121Q | **F**:CTGTGTTCACTTTGGACATGTTG<br>**R**:GACGTTGGAAGATACCAGGTTG | - | Ava II | 238 | 1.5 | 200[b] | 35 | 55 | 97.4 |
| *UCP2* | rs659366 | promoter | -866G>A | - | **F**:AACGTCTTTGGGACTCCGTT<br>**R**:Biotin-GATGAGAAAAGGCGTCAGGA | **F**:TTGGCTGTTCACGC | - | 195 | 1.5[a] | 200[b] | 50 | 55 | 98.3 |
| *FAAH* | rs324420 | coding | 385G>A | P129T | **F**:TGTTGCTGGTTACCCCTCTC<br>**R**:Biotin-CTCACAGGGACGCCATAGAG | **F**:TGAGACTCAGCTGTCTCA | - | 234 | 1.5 | 330[c] | 50 | 55 | 95.9 |
| *KCNJ11*[12] | rs5219 | coding | 67G>A | E23K | **F**:CCGAGAGGACTCTGCAGTGA<br>**R**:CACCAGCGTGGTGAACACGT | - | Ban II | 278 | 3[a] | 200[b] | 40 | 55 | 96.1 |
| *AGTR1* | rs5186 | 3'-UTR | 1166A>C | - | **F**:Biotin-AGAAGCCTGCACCATGTTTT<br>**R**:TGTGGCTTTGCTTTGTCTTG | **R**:CTTCAATTCTGAAAAGTAGC | - | 233 | 1.5 | 330[c] | 50 | 50 | 97.0 |
| *CYP1A1* | rs4646903 | 3'-UTR | 6235T>C | - | **F**:Biotin-CCGCTGCACTTAAGCAGTCT<br>**R**:CATGGTGAAACCCCATCTCT | **R**:GAGAATCGTGTGAGCC | - | 233 | 1.5[a] | 330[c] | 50 | 55 | 94.4 |
| *IRS2*[13] | rs1805097 | coding | 3170G>A | G1057D | **F**:AGGCCTCCTCCCCGTATC<br>**R**:CCATCTCGGTGTAGTCACCA | - | Hae II | 201 | 1.5 | 330[c] | 50 | 55 | 94.8 |

[a]10% DMSO. [b]1x GeneAmp PCR buffer II and 0.5 units of Ampli*Taq* (Applied Biosystems, Nieuwerkerk a/d IJssel, the Netherlands). [c]1x PCR buffer and 0.5 units of *Taq* polymerase (Invitrogen, Breda, the Netherlands). C = cycles, F = forward, GR = genotyping rate, R = reverse, T = annealing temperature.

### 4.3.2 Phenotypes

Zygosity was determined using sequential analysis based on sex, fetal membranes, umbilical cord blood groups, placental alkaline phosphatase and DNA marker analysis. Birth weights were obtained from obstetric records, and gestational age reported by the obstetrician was calculated as the number of completed weeks of pregnancy based on the last menstrual period. Between February 1997 and April 2000, the twins visited the research centre in Leuven for a 2 h examination, which started in the morning after an overnight fast. Participants were measured barefoot and lightly clothed. Standing height (cm) was measured to the nearest 0.1 cm with a Harpenden fixed stadiometer (Holtain, Crosswell, UK). Body mass (kg) was measured on a balance scale (SECA, Hamburg, Germany) to the nearest 0.1 kg. Body mass index (BMI) was calculated as body mass divided by the square of height ($kg/m^2$). Waist and hip circumferences were measured with a flexible steel tape to 1 mm accuracy. Waist circumferences were taken at the smallest point between the costal margin and the iliac crest and hip circumference at the widest part of the hips, generally at the level of the greater trochanters. Waist-to-hip circumference ratio (WHR) was expressed as a percentage. Lean body mass was measured using a bioelectrical impedance analyzer (BIA310; Biodynamics, Seattle, WA, USA). Fat mass (kg) was calculated by subtracting the value for lean body mass from total body mass. Skinfold thicknesses were taken in duplicate, to 0.1 mm accuracy with a Harpenden skinfold calliper (British Indicators, St Albans, UK) at the biceps, triceps, subscapular and suprailiac. The four thicknesses were summed (S4SF) to evaluate the overall subcutaneous fatness. Blood samples were drawn to measure plasma hormone concentrations. Plasma leptin was measured with an immunoradiometric assay in a coated tube (Diagnostic Systems Laboratories, Webster, TX, USA). Plasma lipids (triglycerides, total cholesterol and HDL-cholesterol) were measured on an auto-analyser (AU600; Olympus, Kyoto, Japan). LDL-cholesterol was estimated using Friedewald's formula.[14] Non-esterified fatty acids (NEFA) were measured using a colorimetric assay with the optical density measured at 550 nmol/l. Plasma glucose was measured using the hexokinase method (Olympus AU600). Plasma insulin was determined using a microparticle enzyme immunoassay (Axsym; Abbott Laboratories, Chicago, IL, USA). Insulin-like growth factor binding protein 1 (IGFBP1) was measured by radio-immunoassay, as described.[15]

### 4.3.3 Genotyping

Genomic DNA was initially extracted from available placental tissue collected at birth and/or twins were contacted and whole blood or mouth swabs were taken for DNA extraction. The DNA mini kit (Qiagen, Venlo, the Netherlands) was used for placental and mouth swab DNA extraction, the Wizard kit (Promega, Leiden, the Netherlands) was used for blood DNA extraction. Of the MZ twins, only one member per pair has been genotyped. There is a possibility that placental tissues were switched within twin pairs during collection at birth (33%). As a consequence genotypes and phenotypes would be combined incorrectly. To avoid false combinations, discordant genotypes within DZ pairs, of whom only DNA extracted from placenta tissue was available (64 individuals), were treated as missing values (less than 7% per SNP). Consequently, the total number of genotypes per SNP vary.

In total eleven SNPs were genotyped and the corresponding genes including SNP location, reference number, primer sequences, genotyping assay and genotype rate are described in Table 1. Four SNPs were genotyped using the PCR-RFLP technique according to previously

reported assays in literature (*KCNJ11, ENPP1* and *IRS2,* Table 1) or in house development (*GAD2*). The remaining seven SNP assays were in house designed using Pyrosequencing (PSQ) technology (Pyrosequencing AB, Uppsala, Sweden). PCRs were carried out in 15 µl (PSQ) or 30 µl (RFLP) mixes using 50 ng genomic DNA and 5 (PSQ) or 10 (RFLP) pmol of forward and reverse PCR primers (Eurogentec, Seraing, Belgium), 1x PCR buffer and 0.5 units of *Taq* polymerase. Number of cycles, $MgCl_2$ concentrations, dNTP concentrations, annealing temperature and supplementation of DMSO are listed in Table 1. Further details of PCR cycle times as well as details for the *GAD2* RFLP assay can be obtained from the authors on request.
Primers for the Pyrosequencing assays were designed using Pyrosequencing Primer SNP Design Version 1.01 software (http://techsupport.pyrosequencing.com). Pyrosequencing was performed for sequence determination and allele designation in a Biotage PSQ HS 96A System according to the manufacturers instructions. Prior to genotyping, at least 20 samples were sequenced directly to validate the assays.

### *4.3.4 Statistical analyses*

Phenotypic characteristics of the twin sample are expressed as mean ± SD according to gender and zygosity. Variables with a skewed distribution were transformed to a natural logarithmic scale when performing statistical tests (see Table S1). Differences in means between men and women and MZ and DZ twins were calculated using the PROC MIXED method implemented in the statistical package SAS (version 9.1, SAS Institute). A random intercept model was used, where the intercept of each twin pair was modelled as a function of the population intercept plus an unique contribution of the twin pair. In addition, we allowed the variance-covariance structure of the random intercept to differ between MZ and DZ pairs. For birth weight the intra-pair correlations differ between MZ monochorionic (MC) and MZ dichorionic (DC) twins. Since data on chorionicity were available, the variance-covariance structure of the random intercept of birth weight was allowed to differ between MZ MC, MZ DC and DZ pairs. Differences were considered significant if $p<0.05$. A $\chi^2$-test was used to check Hardy-Weinberg equilibrium for genotype frequencies of the SNPs, using only one randomly selected twin per pair.
Before the association analyses were performed, the effects of potential covariates on all phenotypes were also checked using PROC MIXED. The phenotypes BMI, WHR and S4SF were checked for the effect of zygosity, gender and age; body mass, fat mass and lean body mass were checked for the effect of zygosity, gender, age and height; blood parameters were checked for the effect of zygosity, gender, age and fat (BMI, WHR or S4SF) and birth weight was checked for the effect of gender, chorionicity and gestational age. Covariates were incorporated into the model when the F-test indicated $p<0.10$ and are listed in Table S1. Subsequently, the genotype was entered into the optimised model as a class variable and a general association test (no assumption for mode of inheritance) was performed using a 2 *df* F-test. If the number of subjects homozygous for the minor allele was <10, this group was excluded from the analyses and a 1 *df* F-test was performed. The association analysis was performed on the complete sample, and for men and women separately. An association was considered significant if the *p*-value of the general association test was below the significance threshold defined. The mode of inheritance was investigated by testing an additive, dominant and a recessive model using a 1 *df* F-test.

**Table 2.** Phenotypic characteristics of the twins according to gender and zygosity.

| Characteristic | Sex | | | | Zygosity | | | |
|---|---|---|---|---|---|---|---|---|
| | Men | Women | *p* | d | MZ | DZ | *p* | d |
| n | 286 | 342 | | | 396[a] | 232[a] | | |
| Birth weight (g) | 2596 ± 449 | 2498 ± 486 | 0.0009 | 0.27 | 2494 ± 470 | 2626 ± 464 | 0.006 | 0.23 |
| Gestational age (wks)[b] | 37.1 ± 2.3 | 37.1 ± 2.5 | 0.98 | 0.00 | 36.8 ± 2.4 | 37.5 ± 2.3 | 0.002 | 0.26 |
| Age (yrs)[b] | 25.2 ± 4.6 | 25.1 ± 4.7 | 0.80 | 0.02 | 24.9 ± 4.6 | 25.6 ± 4.7 | 0.08 | 0.14 |
| Body height (cm) | 178.2 ± 6.2 | 165.7 ± 6.5 | <0.0001 | 1.72 | 171.0 ± 8.9 | 172.0 ± 8.9 | 0.29 | 0.09 |
| Body mass (kg) | 69.5 ± 9.3 | 60.2 ± 9.5 | <0.0001 | 0.85 | 64.3 ± 10.6 | 65.0 ± 10.3 | 0.79 | 0.02 |
| BMI (kg/m$^2$)[c] | 21.7 ± 1.1 | 21.7 ± 1.1 | 0.85 | 0.02 | 21.7 ± 1.1 | 21.6 ± 1.1 | 0.73 | 0.03 |
| Fat mass (kg)[c] | 11.6 ± 1.5 | 16.6 ± 1.3 | <0.0001 | 0.83 | 14.0 ± 1.5 | 14.4 ± 1.4 | 0.45 | 0.06 |
| Lean body mass (kg) | 56.9 ± 6.2 | 42.9 ± 5.3 | <0.0001 | 2.06 | 49.2 ± 9.1 | 49.4 ± 9.0 | 0.79 | 0.02 |
| S4SF (mm)[c] | 34.5 ± 1.5 | 54.9 ± 1.4 | <0.0001 | 1.07 | 45.3 ± 1.6 | 43.0 ± 1.5 | 0.27 | 0.09 |
| WHR (%) | 82.7 ± 5.2 | 72.8 ± 4.3 | <0.0001 | 1.91 | 77.6 ± 6.8 | 76.9 ± 6.8 | 0.31 | 0.09 |
| IGFBP1 (ng/ml)[c] | 11.4 ± 1.8 | 17.3 ± 2.1 | <0.0001 | 0.55 | 13.6 ± 2.0 | 15.6 ± 2.1 | 0.05 | 0.16 |
| Fasting insulin (pmol/l)[c] | 32.2 ± 1.5 | 38.8 ± 1.5 | <0.0001 | 0.41 | 35.3 ± 1.6 | 36.1 ± 1.5 | 0.59 | 0.05 |
| Fasting glucose (mmol/l) | 4.9 ± 0.4 | 4.6 ± 0.4 | <0.0001 | 0.73 | 4.8 ± 0.4 | 4.7 ± 0.4 | 0.03 | 0.18 |
| Leptin (ng/ml)[c] | 1.6 ± 3.0 | 11.7 ± 2.0 | <0.0001 | 1.95 | 4.6 ± 4.0 | 4.5 ± 3.7 | 0.81 | 0.02 |
| Total cholesterol (mmol/l) | 4.8 ± 1.0 | 5.2 ± 0.9 | <0.0009 | 0.27 | 5.0 ± 0.9 | 5.1 ± 1.0 | 0.10 | 0.14 |
| LDL-cholesterol (mmol/l) | 3.0 ± 1.0 | 2.9 ± 0.8 | 0.03 | 0.18 | 2.9 ± 0.9 | 3.0 ± 0.9 | 0.25 | 0.10 |
| HDL-cholesterol (mmol/l) | 1.4 ± 0.3 | 1.8 ± 0.4 | <0.0001 | 1.07 | 1.6 ± 0.4 | 1.6 ± 0.5 | 0.32 | 0.08 |
| Triglycerides (mmol/l)[c] | 0.8 ± 1.5 | 0.9 ± 1.5 | 0.18 | 0.11 | 0.9 ± 1.5 | 0.9 ± 1.5 | 0.61 | 0.04 |
| NEFA (mmol/l)[c] | 0.5 ± 1.5 | 0.6 ± 1.3 | <0.0001 | 0.74 | 0.6 ± 1.5 | 0.6 ± 1.5 | 0.60 | 0.04 |

Data are expressed as mean ± SD. [a]MZ: 179 females and 217 males; DZ: 125 females and 107 males. [b]*p*-value and effect size calculated using standard linear regression, because convergence criteria could not be met in the random intercept model. [c]Geometric mean ± SD. d = Cohen's d effect size (d=ABS($LSMean_1$-$LSMean_2$)/(SE/SQRT((1/$n_1$)+(1/$n_2$))) where SE = standard error of the difference calculated using random intercept model, LS = least squares, ABS = absolute value, SQRT = square root); 0.2 indicates a small effect size, 0.5 medium and 0.8 large.[16]

**Table 3.** Genotype and allele frequencies of the SNPs in the twin sample.

| | | Genotype | | | Allele | |
|---|---|---|---|---|---|---|
| | | Homozygous major allele[a] ($n_M$/$n_W$) | Heterozygous[a] ($n_M$/$n_W$) | Homozygous minor allele[a] ($n_M$/$n_W$) | Major allele[a] | Minor allele[a] |
| *PPARγ* | P12A | 0.794 (221/259) | 0.199 (52/68) | 0.007 (3/1) | 0.894 | 0.106 |
| *IL6* | -174G>C | 0.340 (106/101) | 0.470 (121/154) | 0.190 (48/58) | 0.575 | 0.425 |
| *GAD2* | -243A>G | 0.672 (175/229) | 0.293 (96/82) | 0.035 (9/14) | 0.818 | 0.182 |
| *PYY* | R72T | 0.407 (116/124) | 0.474 (122/171) | 0.119 (36/37) | 0.644 | 0.356 |
| *ENPP1* | K121Q | 0.800 (226/260) | 0.190 (50/71) | 0.01 (2/3) | 0.895 | 0.105 |
| *UCP2* | -866G>A | 0.432 (101/158) | 0.467 (145/134) | 0.101 (28/33) | 0.666 | 0.334 |
| *FAAH* | P129T | 0.673 (170/227) | 0.301 (88/95) | 0.026 (11/6) | 0.824 | 0.176 |
| *KCNJ11* | E23K | 0.395 (99/136) | 0.467 (126/151) | 0.138 (42/37) | 0.628 | 0.372 |
| *AGTR1* | 1166A>C | 0.476 (125/166) | 0.450 (127/137) | 0.074 (25/22) | 0.701 | 0.299 |
| *CYP1A1* | 6235T>C | 0.851 (244/273) | 0.143 (32/48) | 0.006 (1/3) | 0.922 | 0.078 |
| *IRS2* | G1057D | 0.407 (108/128) | 0.480 (127/159) | 0.113 (32/33) | 0.647 | 0.353 |

[a]Frequencies are calculated using a sub-sample, that only included one randomly selected twin per pair. See Table S3 of the online appendix for the numbers per genotype group of the sub-sample. $n_M$ = number of men in the whole twin sample, $n_W$ = number of women in the whole twin sample.

### 4.3.5 Multiple testing

Since multiple tests were performed, a principal component factor analysis (PCFA) was carried out on all phenotypes to assess the number of separate hypotheses that were actually tested. The PCFA was performed on residuals adjusted for significant covariates and the number of factors was selected based on a minimal eigenvalue of 1.0. To produce interpretable factors we used an orthogonal Varimax rotation, since oblique rotation (i.e. Oblimin) showed that the inter-factor correlations were low (<0.10). In addition, the factor loadings yielded by oblique rotation were very similar to those produced by the Varimax rotation. Variables sharing at least 50% of their variance with a factor, equivalent to a factor loading of >0.70, were considered as measuring the same construct and were included in the factor.
In total six factors were extracted by PCFA (Table S2). Factor 1 contained lean body mass and four obesity parameters, respectively body mass, BMI, fat mass and S4SF. Factor 2 showed high loadings for total cholesterol and LDL-cholesterol. Factor 3 contained IGFBP1 and fasting glucose levels, and factor 4 fasting insulin and leptin levels. Factor 5 showed high loadings with HDL-cholesterol only, and in factor 6 no traits had a factor loading >0.70 (Table S2).
Within each factor, the number of phenotypes ($n_v$) was summed and to determine the significance threshold for these phenotypes a Bonferroni correction was performed ($\alpha/n_v$, where $\alpha$=0.05). Consequently, the significance threshold applied in the association analysis for body mass, BMI, fat mass, lean body mass and S4SF was 0.01, and for total cholesterol, LDL-cholesterol, IGFBP1, fasting glucose, fasting insulin and leptin levels 0.025. The remaining phenotypes preserved the significance threshold of 0.05 (Table S2).

## 4.4 RESULTS

### 4.4.1 Descriptive statistical analysis

Phenotypic characteristics of the twins are summarised by gender and zygosity in Table 2. Men had higher weight at birth and were taller and heavier at adult age than women. In addition, men had lower fat mass, S4SF, IGFBP1, fasting insulin, leptin, total cholesterol, HDL-cholesterol and NEFA levels than women, but had higher lean body mass, WHR, fasting glucose and LDL-cholesterol levels ($p$<0.05). MZ twins had a lower birth weight, shorter gestational age and higher fasting glucose levels at adult age than DZ twins ($p$<0.05).
Genotype frequencies of all SNPs (Table 3) were in accordance with Hardy-Weinberg equilibrium ($p$>0.05).

### 4.4.2 General association analysis

The results of the association analyses are presented in Tables 4 and 5 for the complete sample ($p_{All}$) and for men ($p_M$) and women ($p_W$) separately. The means and 95% confidence intervals (CI) per genotype group and the $p$-values of the different inheritance models of the SNPs that showed a significant association with a certain phenotype (bold and underlined in Table 4 and 5) are presented in Table 6. The SNPs in *KCNJ11, AGTR1*, *CYP1A1* and *IRS2* did not show significant association (Table 5).

**Table 4.** Association between T2D related metabolic risk factors and SNPs in *PPARγ*, *IL6*, *GAD2*, *PYY*, *ENPP1* and the *UCP2* gene adjusted for significant covariates carried out on the complete, men-only and women-only samples.

| | | | *PPARg* P12A | | | *IL-6* -174G>C | | | *GAD2* -243A>G | | | *PYY* R72T | | | *ENPP1* K121Q | | | *UCP2* -866G>A | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Trait | F | α | $p_{All}$[a] | $p_M$[a] | $p_W$[a] | $p_{All}$ | $p_M$ | $p_W$ | $p_{All}$ | $p_M$[a] | $p_W$ | $p_{All}$ | $p_M$ | $p_W$ | $p_{All}$[a] | $p_M$[a] | $p_W$[a] | $p_{All}$ | $p_M$ | $p_W$ |
| Birth weight | | 0.05 | **0.04** | 0.19 | 0.11 | 0.57 | 0.64 | 0.80 | 0.90 | 0.22 | 0.99 | 0.12 | 0.32 | 0.26 | 0.45 | 0.76 | 0.55 | 0.89 | 0.39 | 0.72 |
| Body mass | 1 | 0.01 | 0.25 | 0.42 | 0.14 | 0.29 | 0.15 | 0.03 | 0.28 | 0.03 | 0.44 | 1.00 | 0.92 | 0.83 | 0.46 | 0.18 | 0.92 | 0.94 | 0.73 | 0.29 |
| BMI | 1 | 0.01 | 0.21 | 0.58 | 0.20 | 0.24 | 0.19 | 0.02 | 0.40 | 0.02 | 0.48 | 0.98 | 0.93 | 0.77 | 0.43 | 0.23 | 0.97 | 0.69 | 0.64 | 0.35 |
| Fat mass | 1 | 0.01 | 0.03 | 0.05 | 0.06 | 0.35 | 0.51 | 0.02 | 0.78 | 0.92 | 0.57 | 0.25 | 0.64 | 0.54 | 0.61 | 0.85 | 0.75 | 0.64 | 0.73 | 0.46 |
| Lean body mass | 1 | 0.01 | 0.77 | 0.31 | 0.41 | 0.32 | 0.09 | 0.06 | 0.05 | **0.004** | 0.54 | 0.61 | 0.64 | 0.97 | 0.17 | 0.03 | 0.76 | 0.20 | 0.22 | 0.18 |
| S4SF | 1 | 0.01 | 0.11 | 0.16 | 0.25 | 0.06 | 0.88 | **0.005** | 0.40 | 0.42 | 0.61 | 0.68 | 0.88 | 0.51 | 0.89 | 0.18 | 0.30 | 0.71 | 0.85 | 0.29 |
| WHR | | 0.05 | **0.03** | 0.22 | 0.06 | 0.55 | 0.80 | 0.58 | 0.33 | 0.60 | 0.75 | 0.59 | 0.33 | 0.91 | 0.91 | 0.89 | 0.73 | 0.68 | 0.44 | 0.23 |
| IGFBP1 | 3 | 0.025 | 0.65 | 0.42 | 0.91 | 0.25 | 0.52 | 0.35 | 0.82 | 0.55 | 0.55 | 0.36 | 0.54 | 0.54 | 0.93 | 0.24 | 0.49 | 0.32 | 0.03 | 0.99 |
| Fasting glucose | 3 | 0.025 | 0.28 | 0.07 | 0.92 | 0.07 | 0.38 | 0.08 | 0.63 | 0.13 | 0.61 | 0.62 | 0.07 | **0.01** | 0.18 | 0.25 | 0.97 | 0.67 | 0.45 | 0.72 |
| Fasting insulin | 4 | 0.025 | 0.18 | 0.08 | 0.90 | 0.28 | 0.13 | 0.87 | **0.01** | **0.004** | 0.12 | 0.55 | 0.87 | 0.56 | 0.98 | 0.63 | 0.64 | 0.76 | 0.58 | 0.19 |
| Leptin | 4 | 0.025 | 0.67 | 0.05 | 0.20 | 0.46 | 0.24 | 0.11 | 0.94 | 0.57 | 0.66 | 0.45 | 0.87 | 0.25 | 0.65 | 0.84 | 0.52 | 0.98 | 0.95 | 0.91 |
| Total cholesterol | 2 | 0.025 | 0.48 | 0.29 | 0.69 | 0.88 | 0.51 | 0.81 | 0.63 | 0.13 | 0.33 | 0.87 | 0.15 | 0.16 | 0.53 | 0.81 | 0.72 | 0.49 | 0.37 | 0.05 |
| LDL-cholesterol | 2 | 0.025 | 0.99 | 0.83 | 0.91 | 0.88 | 0.86 | 0.55 | 0.68 | 0.38 | 0.30 | 0.85 | 0.31 | 0.15 | 0.09 | 0.48 | 0.12 | 0.40 | 0.27 | **0.01** |
| HDL-cholesterol | 5 | 0.05 | 0.44 | 0.15 | 0.77 | **0.03** | 0.20 | 0.29 | 0.06 | 0.42 | 0.26 | 0.35 | 0.20 | 0.68 | 0.10 | 0.78 | **0.05** | 0.89 | 0.96 | 0.78 |
| Triglycerides | | 0.05 | 0.98 | 0.52 | 0.49 | 0.11 | 0.68 | **0.05** | 0.25 | 0.14 | 0.08 | 0.42 | 0.68 | 0.12 | 0.66 | 0.97 | 0.57 | 0.87 | 0.14 | 0.49 |
| NEFA | | 0.05 | 0.41 | 0.80 | 0.10 | 0.33 | **0.02** | 0.45 | 0.09 | 0.87 | 0.14 | 0.87 | 0.32 | 0.12 | 0.49 | **0.02** | 0.17 | 0.93 | 0.55 | 0.46 |

[a]Homozygous major allele vs heterozygous only (homozygous minor allele group not included, because it contained less then 10 individuals). α = significance threshold, F = factor, $p_{All}$ = *p*-value complete analysis, $p_M$ = *p*-value men-only analysis, $p_W$ = *p*-value women-only analysis. *p*-values lower than significance threshold are shown in **boldface type and underlined**.

**Table 5.** Association between T2D related metabolic risk factors and SNPs in *FAAH, KCNJ11*, *AGTR1*, *CYP1A1* and the *IRS2* gene adjusted for significant covariates carried out on the complete, men-only and women-only samples.

| | | | *FAAH* P129T | | | *KCNJ11* E23K | | | *AGTR1* 1166A>C | | | *CYP1A1* 6235T>C | | | *IRS2* G1057D | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Trait | F | α | $p_{All}$ | $p_M$ | $p_W$[a] | $p_{All}$ | $p_M$ | $p_W$ | $p_{All}$ | $p_M$ | $p_W$ | $p_{All}$[a] | $p_M$[a] | $p_W$[a] | $p_{All}$ | $p_M$ | $p_W$ |
| Birth weight | | 0.05 | 0.10 | **0.03** | 0.44 | 0.25 | 0.49 | 0.55 | 0.69 | 0.15 | 0.15 | 0.90 | 0.38 | 0.92 | 0.06 | 0.08 | 0.72 |
| Body mass | 1 | 0.01 | 0.53 | 0.67 | 0.34 | 0.49 | 0.20 | 0.72 | 0.35 | 0.26 | 0.98 | 0.05 | 0.06 | 0.28 | 0.84 | 0.38 | 0.58 |
| BMI | 1 | 0.01 | 0.35 | 0.78 | 0.21 | 0.49 | 0.16 | 0.91 | 0.29 | 0.24 | 0.96 | 0.04 | 0.02 | 0.36 | 0.74 | 0.32 | 0.51 |
| Fat mass | 1 | 0.01 | 0.82 | 0.55 | 0.60 | 0.37 | 0.56 | 0.92 | 0.30 | 0.16 | 0.57 | 0.15 | 0.31 | 0.47 | 0.67 | 0.67 | 0.19 |
| Lean body mass | 1 | 0.01 | 0.15 | 0.49 | 0.08 | 0.40 | 0.44 | 0.67 | 0.10 | 0.04 | 0.76 | 0.06 | 0.08 | 0.16 | 0.42 | 0.27 | 0.86 |
| S4SF | 1 | 0.01 | 0.79 | 0.61 | 0.93 | 0.36 | 0.28 | 0.79 | 0.37 | 0.19 | 0.71 | 0.10 | 0.23 | 0.26 | 1.00 | 0.44 | 0.36 |
| WHR | | 0.05 | 0.37 | 0.73 | 0.19 | 0.55 | 0.08 | 0.43 | 0.52 | 0.61 | 0.89 | 0.14 | 0.22 | 0.38 | 0.42 | 0.38 | 0.96 |
| IGFBP1 | 3 | 0.025 | 0.26 | 0.78 | 0.17 | 0.69 | 0.81 | 0.80 | 0.15 | 0.66 | 0.24 | 0.90 | 0.82 | 0.67 | 0.31 | 0.88 | 0.24 |
| Fasting glucose | 3 | 0.025 | 0.76 | 0.38 | 0.30 | 0.10 | 0.07 | 0.29 | 0.85 | 0.62 | 0.62 | 0.92 | 0.26 | 0.73 | 0.05 | 0.10 | 0.20 |
| Fasting insulin | 4 | 0.025 | 0.89 | 0.35 | 0.44 | 0.42 | 0.21 | 0.90 | 0.86 | 0.97 | 0.73 | 0.16 | 0.69 | 0.38 | 0.82 | 0.19 | 0.07 |
| Leptin | 4 | 0.025 | 0.29 | 0.49 | 0.56 | 0.06 | 0.04 | 0.90 | 0.95 | 0.48 | 0.84 | 0.17 | 0.09 | 0.41 | 0.30 | 0.03 | 0.19 |
| Total cholesterol | 2 | 0.025 | 0.40 | 0.32 | 0.92 | 0.72 | 0.61 | 0.72 | 0.89 | 0.22 | 0.54 | 0.30 | 0.15 | 0.95 | 0.88 | 0.62 | 0.70 |
| LDL-cholesterol | 2 | 0.025 | 0.39 | 0.78 | 0.20 | 0.81 | 0.27 | 0.18 | 0.88 | 0.14 | 0.22 | 0.33 | 0.13 | 0.73 | 0.79 | 0.77 | 0.44 |
| HDL-cholesterol | 5 | 0.05 | 0.21 | 0.46 | 0.07 | 0.10 | 0.11 | 0.63 | 0.72 | 0.23 | 0.62 | 0.61 | 0.30 | 0.68 | 0.70 | 0.48 | 0.94 |
| Triglycerides | | 0.05 | 0.29 | 0.06 | 0.42 | 0.75 | 0.77 | 0.35 | 0.51 | 0.06 | 0.96 | 0.61 | 0.08 | 0.30 | 0.94 | 0.96 | 0.86 |
| NEFA | | 0.05 | 0.84 | 0.82 | 0.26 | 0.34 | 0.12 | 0.85 | 0.56 | 0.12 | 0.62 | 0.38 | 0.17 | 0.78 | 0.14 | 0.65 | 0.14 |

[a]Homozygous major allele vs heterozygous only (homozygous minor allele group not included, because it contained less then 10 individuals). α = significance threshold, F = factor, $p_{All}$ = *p*-value complete analysis, $p_M$ = *p*-value men-only analysis, $p_W$ = *p*-value women-only analysis. *p*-values lower than significance threshold are shown in **boldface type and underlined**.

In the complete sample, A allele carriers of the *PPARγ* P12A SNP had higher birth weight ($p_G=p_A$=0.04, α=0.05, effect size (d) =0.21) and higher WHR ($p_D$=0.02, α=0.05, d=0.23). CC carriers of the *IL6* -174G>C SNP had higher HDL-cholesterol levels ($p_R$=0.008, α=0.05, d=0.29), and the promoter SNP in *GAD2* (-243A>G) was associated with fasting insulin levels ($p_G$=0.01, α=0.025, d=0.21/0.59) (Table 6).

### *4.4.3 Sex-specific association analysis*

In men only, CC carriers of the *IL6* -174G>C SNP had higher NEFA levels ($p_R$=0.005, α=0.05, d=0.46). The G allele of the *GAD2* -243A>G SNP was associated with lower lean body mass ($p_G=p_A$=0.004, α=0.01, d=0.38) and lower fasting insulin levels ($p_G=p_A$=0.004, α=0.025, d=0.37). Men carrying the Q allele of the K121Q SNP of *ENPP1* had lower NEFA levels ($p_G=p_A$=0.02, α=0.05, d=0.38), and the T allele of the *FAAH* P129T SNP was associated with a higher weight at birth ($p_A$=0.02, α=0.05, d=0.16/0.61) (Table 6).
In women only, C allele carriers of the *IL6* (-174G>C) SNP had higher S4SF ($p_D$=0.002, α=0.01, d=0.39) and lower triglycerides levels ($p_R$=0.04, α=0.05, d=0.31). Furthermore, the TT genotype of the *PYY* R72T SNP was associated with higher fasting glucose levels ($p_R$=0.003, α=0.025, d=0.36), and Q allele carriers of the *ENPP1* K121Q SNP had higher HDL-cholesterol levels ($p_G=p_A$=0.05, α=0.05, d=0.27). Finally, A allele carriers of the *UCP2* -866G>A SNP had higher LDL-cholesterol levels ($p_D$=0.01, α=0.025, d=0.30).

## 4.5 DISCUSSION

In this study we examined whether SNPs, that have previously been associated with T2D or disease-related traits, are associated with birth weight or adult metabolic risk factors measured in young Belgian twins. Association analyses were carried out on the complete sample and for men and women separately. Although a few SNPs were associated with metabolic risk factors in the complete sample, the majority of the detected associations were sex-specific.

***PPARγ* (P12A).** In the complete sample, the A allele of the P12A SNP in *PPARγ*, a transcription factor involved in adipocyte differentiation, lipid storage and glucose metabolism, was associated with a higher birth weight and WHR. Currently, the A allele of the P12A SNP is generally regarded as protective for T2D,[8] which seems in disagreement with our results. However, a large meta-analysis (n=32,000) reported a significantly increased BMI in non-diabetic Caucasian A allele carriers (Table S5).[17] The opposing associations of the P12A SNP in T2D and obesity may be explainable by a second SNP in *PPARγ* (C1431T). Because the protective effect of the A allele apparently disappears in cases where the A allele is on the same chromosome as the T allele of the C1431T SNP that has been associated with an increased BMI, but not with insulin indices.[18,19] Conflicting results have also been published concerning the P12A SNP and its association with birth weight (Table S5). Pihlajamaki et al.[20] reported a significantly higher ponderal index and a trend for higher birth weight in A allele carriers. However, Pfab et al.[21] and Labayen et al.[22] failed to replicate these findings. Our results support the findings of Pihlajamaki et al.[20] and suggest that P12A SNP is involved in the regulation of birth weight. However, minor allele frequency and sample size are too small to draw definite conclusions at this point.

**Table 6.** Means (95% CI) per genotype group and mode of inheritance (additive, dominant and recessive) of the significant associations.

| *Gene* (SNP) | Trait[a] | S | Genotype | | | $p_G$ | $p_A$ | $p_D$ | $p_R$ | d |
|---|---|---|---|---|---|---|---|---|---|---|
| *PPARγ* (P12A) | | | PP | PA | AA | | | | | |
| | Birth weight | A | 2528 (2487-2569) (474) | 2614 (2537-2691) (118) | 2339 (1984-2695) (4)[b] | **0.04**[c] | **0.04**[c] | 0.07 | - | 0.21 |
| | WHR | A | 77.3 (76.8-77.8) (473) | 78.4 (77.5-79.3) (120) | 80.3 (75.9-84.7) (4)[b] | 0.03[c] | 0.03[c] | **0.02** | - | 0.23 |
| *IL6* (-174G>C) | | | GG | GC | CC | | | | | |
| | HDL-cholesterol | A | 1.60 (1.54-1.66) (197) | 1.59 (1.54-1.64) (262) | 1.71 (1.63-1.79) (101) | 0.03 | 0.05 | 0.49 | **0.008** | 0.29 |
| | NEFA[d] | M | 0.46 (0.43-0.50) (103) | 0.47 (0.43-0.50) (119) | 0.56 (0.50-0.64) (48) | 0.02 | 0.03 | 0.30 | **0.005** | 0.46 |
| | S4SF[d] | W | 49.1 (45.6-53.0) (98) | 57.8 (54.4-61.4) (152) | 54.7 (49.7-60.1) (57) | 0.005 | 0.03 | **0.002** | 0.84 | 0.39 |
| | Triglycerides[d] | W | 0.87 (0.79-0.95) (92) | 0.95 (0.88-1.02) (145) | 0.79 (0.70-0.90) (55) | 0.05 | 0.46 | 0.54 | **0.04** | 0.31 |
| *GAD2* (-243A>G) | | | AA | AG | GG | | | | | |
| | Fasting insulin[d] | A | 36.4 (34.8-38.1) (394) | 33.1 (30.9-35.5) (169) | 43.7 (35.7-53.6) (21) | **0.01** | 0.48 | 0.11 | 0.05 | 0.21/0.59[e] |
| | Lean body mass | M | 57.6 (56.7-58.5) (173) | 55.4 (54.2-56.6) (94) | 58.5 (54.5-62.5) (9)[b] | **0.004**[c] | **0.004**[c] | 0.008 | **-** | 0.38 |
| | Fasting insulin[d] | M | 34.0 (31.8-36.4) (174) | 28.8 (26.3-31.6) (93) | 36.1 (26.6-48.9) (9)[b] | **0.004**[c] | **0.004**[c] | 0.01 | **-** | 0.37 |
| *PYY* (R72T) | | | RR | RT | TT | | | | | |
| | Fasting glucose | W | 4.55 (4.47-4.62) (121) | 4.53 (4.47-4.60) (161) | 4.76 (4.63-4.90) (35) | 0.01 | 0.06 | 0.55 | **0.003** | 0.36 |
| *ENPP1* (K121Q) | | | KK | KQ | QQ | | | | | |
| | NEFA[d] | M | 0.49 (0.46-0.52) (222) | 0.41 (0.36-0.47) (49) | 0.86 (0.53-1.41) (2)[b] | **0.02**[c] | **0.02**[c] | 0.06 | - | 0.38 |
| | HDL-cholesterol[d] | W | 1.77 (1.71-1.83) (242) | 1.89 (1.78-2.01) (68) | 1.39 (1.05-1.84) (3)[b] | **0.05**[c] | **0.05**[c] | 0.11 | - | 0.27 |
| *UCP2* (-866G>A) | | | GG | GA | AA | | | | | |
| | LDL-cholesterol | W | 2.77 (2.63-2.91) (149) | 3.07 (2.92-3.22) (124) | 2.83 (2.51-3.14) (29) | 0.01 | 0.07 | **0.01** | 0.67 | 0.30 |
| *FAAH* (P129T) | | | PP | PT | TT | | | | | |
| | Birth weight | M | 2558 (2492-2625) (170) | 2626 (2538-2715) (88) | 2872 (2630-3115) (11) | 0.03 | **0.02** | 0.07 | 0.02 | 0.16/0.61[e] |

Data are expressed as least squares (LS) means (95% confidence intervals) (number of individuals). Total numbers of individuals per genotype group might differ from numbers reported in Table 3, because of missing values for dependent variable or covariates. [a]Units of the phenotypes are listed in Table 2. [b]Not included in general, additive and recessive association analysis, because genotype group <10 individuals. [c]Homozygous major allele *vs* heterozygous only. A = all individuals, M = men-only, $p_G$ = *p*-value general association model, $p_A$ = *p*-value additive model, $p_D$ = *p*-value dominant model, $p_R$ = *p*-value recessive model, S = sample, W = women-only. d = Cohen's d effect size ($d=ABS(LSMean_1-LSMean_2)/(SE/SQRT((1/n_1)+(1/n_2)))$; 0.2 indicates a small effect size, 0.5 medium and 0.8 large. [d]Geometric LSMean (95% CI). [e]Homozygous major allele *vs* heterozygous / heterozygous *vs* homozygous minor allele. The lowest *p*-value is shown in **boldface type and underlined**. See supplementary material I Table S4 for the LSmeans (95% CI) per genotype group of the associations that were considered non-significant.

***IL6* (-174G>C).** A large meta-analysis (n>20,000) reported that the C allele of the *IL6* –174G>C SNP was associated with a reduced T2D risk (OR=0.91).[23] Conversely, studies published on the role of the –174G>C SNP in obesity and the lipid metabolism are highly inconsistent (Table S6). We observed in the complete sample that CC carriers had higher HDL-cholesterol, and CC carrying women had lower triglycerides levels, indicating that the C allele might also be protective against cardiovascular disease. However, CC carrying men and women had higher NEFA levels and larger S4SF, respectively, which does not support a protective role. A possible explanation could be that the lipolysis stimulating effect of interleukin-6 reported by van Hall et al.[24] is increased in C carriers. During lipolysis triglycerides are broken down into glycerol and fatty acids, and in our sample this is translated into lower triglycerides levels in women and higher NEFA levels in men carrying the CC genotype. This explanation is highly speculative, because the effect of the –174G>C SNP on interleukin-6 levels (Table S6) and the biological effect of this pro-inflammatory cytokine on insulin sensitivity, are still controversial.[25,26] Nevertheless, in the present analysis the *IL6* –174G>C SNP showed very promising association with several metabolic risk factors, which definitely encourages additional research.

***GAD2* (–243A>G).** *GAD2*, expressed in the brain and pancreatic islets, encodes the glutamic acid decarboxylase enzyme (GAD65) that catalyses the formation of the neurotransmitter γ-aminobutyric acid (GABA). Studies in rodents have suggested that GABA regulates food intake and inhibits glucose-induced insulin release in pancreatic ß cells.[27,28] In our study, heterozygote carriers of the *GAD2* SNP had in the complete sample significantly lower fasting insulin levels than either homozygote carriers or non-carriers. This heterozygosity effect might reflect antagonistic pleiotropic gene action, where a single gene influences multiple traits in both beneficial and detrimental ways. However, despite studies in model organisms suggest otherwise,[29,30] overdominance and underdominance are believed to be uncommon features. Accordingly, it is not very common to evaluate this inheritance pattern in association studies and probably for that reason examples in literature are scarce. Whether our finding is true or false needs to be further investigated, but a recently published association study in Danish subjects (n=5857) reported that the G allele was associated with lower fasting glucose levels under an additive mode of inheritance.[31] In the men-only sample, the G allele of the *GAD2* SNP was significantly associated with lower fasting insulin levels and lower lean body mass ($p$=0.004). The latter was accompanied with a trend towards association with lower body mass ($p$=0.03) and BMI ($p$=0.02) (Table S4). Because of the low minor allele frequency, it was not possible to discriminate between the general association and the additive model, and therefore the mode of inheritance remains unclear. Nevertheless, the direction of the effect we observe in the men-only sample is in conflict with the French study that originally reported an association between the –243A>G *GAD2* SNP and obesity,[32] but is in accordance with later large studies in Danish and British subjects reporting a lower BMI in G-allele carriers (Table S7).[31,33] Interestingly, our results suggest that the lower BMI associated with the G allele is attributed to a lower lean body mass.

***PYY* (R72T).** The gut hormone PYY, which exits in two forms i.e. $PYY_{1-36}$ and $PYY_{3-36}$, is well-known for its food intake suppressing effect.[34] Several reports studying the association between the *PYY* R72T SNP and obesity or related traits reported lack of association (Table S8). We observed a sex-specific association, with R allele carrying women having lower fasting glucose levels. This is in disagreement with Torekov et al.[35] who reported in Danish whites that the R allele was associated with T2D and increased plasma glucose levels 2 h

after an oral glucose tolerance test. A possible explanation for this inconsistency might be the difference in BMI of our sample (mean BMI=21.8) compared to the Danish sample (mean BMI=26.3), since Sloth et al.[36] observed that after $PYY_{1-36}$ injections appetite ratings were different among lean and obese persons. They speculated that this could be due to different expression of the neuropeptide Y1, Y2 and Y5 receptors that have opposite effects on appetite, with Y2 having anorexigenic effects, and Y1 and Y5 orexigenic effects.[36]

***ENPP1* (K121Q).** For the K121Q SNP in *ENPP1*, also known as plasma cell membrane glycoprotein 1 (*PC-1*) that impairs insulin signalling by interacting with the insulin receptor, a large meta-analysis reported that the QQ genotype was associated with an increased T2D risk (OR=1.38).[37] This effect appears to be modulated by obesity since it disappeared after adjusting for BMI. In addition, Costanzo et al.[38] showed that the Q variant inhibits signalling of the insulin receptor more effective than the K variant. These findings appear to be in conflict with our results, where men carrying the Q allele had lower NEFA levels and women higher HDL-cholesterol levels. In Table S9, we summarised the studies published on the *ENPP1* K121Q SNP in relation to the metabolic traits examined in our study. This table shows that the few studies reporting a significant relation between the *ENPP1* K121Q SNP and lipid parameters, reported an effect in the opposite direction and therefore our results on this SNP are likely to represent false-positive associations.

***UCP2* (-866G>A).** The A allele of the -866G>A SNP in *UCP2*, belonging to the mitochondrial inner membrane carrier protein family that diminishes ATP production by causing a proton leak, has initially been associated with enhanced adipose tissue mRNA expression and decreased obesity risk.[39] In contrast, the A allele has also frequently been associated with increased T2D risk (Table S10), which is thought to be the result of an increased UCP2 expression in β-cells, causing reduced ATP production, and accordingly lower glucose-stimulated insulin secretion.[40] We observed that women carrying the A allele had higher LDL-cholesterol levels, which is in concordance with Reis et al.[41] who observed in T2D patients that A allele carriers had higher triglycerides, total and LDL-cholesterol levels.[41] In addition, a large study in men (n=2695) reported that AA homozygotes had a 2-fold increase in coronary heart disease risk,[42] and Oberkofler et al.[43] observed that women carrying the A allele had an increased risk on asymptomatic carotid atherosclerosis. Opposed to adipose tissue, in endothelial cells and macrophages the G allele has been related to increased transcription rates, which has been suggested to reduce the generation of reactive oxygen species, and thereby protecting against oxidative damage and atherosclerosis.[43] Our results suggest that the reported association between the minor A allele and increased cardiovascular disease risk,[42,43] might partially be explained by higher basal LDL-cholesterol levels in A allele carriers, since oxidation of LDL-cholesterol in the vascular endothelium is a precursor to plaque formation.

***FAAH* (P129T).** FAAH inactivates endogenous cannabinoid receptor ligands that, among other activities, stimulate feeding behaviour.[44] Thus far, one study reported that the T allele was associated with a decreased obesity risk, and two studies reported an increased obesity risk (Table S11). The latter results are supported by *in vitro* studies in T-lymphocytes and COS-7 cells demonstrating that the T allele variant was characterised by a reduced cellular expression and activity.[45] We observed an association between the T allele and higher birth weight in men. This is unlikely the result of increased intra-uterine food intake, but probably reflects actions of the endogenous cannabinoid system on peripheral lipogenesis.[44]

Prior to menopause, women have a better cardiovascular risk profile than men, with higher HDL-cholesterol, and lower LDL-cholesterol and blood pressure levels.[46] In addition, it has been shown that genes are differently expressed among men and women.[47] Hence, it is important to take these sex-specific differences into consideration. Conducting sex-specific association analyses is not very common, probably due to the obvious loss of power. In our analysis this feature was visible for the *PPARγ* P12A SNP that only showed association in the complete sample. This suggests that the effect is present in both sexes, but due to the lower power no evidence was observed in the sub-samples. The *GAD2* –243A>G SNP showed in the complete and the men-only sample association with fasting insulin levels. This suggests that the association observed in the entire sample is a result of the sex-specific association. These sex-specific associations might have implications for the interpretation of studies that did not take them into consideration, but additional studies need to be carried out to confirm them. In addition, because of the small sample size the sex-specific association analyses are susceptible for spurious associations and therefore the results should be interpreted carefully.

For the SNPs in *KCNJ11, AGTR1*, *CYP1A1* and *IRS2* no significant associations were observed. The E23K SNP of *KCNJ11*, which is involved in insulin secretion, is currently considered as a true genetic risk factor of T2D.[7] The negative results for *KCNJ11* in this study might be explained by the lack of insulin secretion parameters, since fasting insulin levels reflect insulin resistance. The *AGTR1* A1166C SNP has mainly been studied in relation to hypertension, but Abdollahi et al.[48] reported association between the *AGTR1* A1166C SNP and several metabolic traits (Table S13). Even though our sample size was bigger we could not replicate their observations, which might be due to the young age of our sample. The *CYP1A1* 6235T>C SNP is a genetic risk factor for lung cancer, but has also been associated with birth weight in Chinese[49] (Table S14). Although the size of our sample is comparable with the Chinese sample, we did not replicate their association. This might suggest that the association observed in the Chinese sample represents an ethnic-specific effect. Also for the G1057D SNP in the *IRS2* gene that plays a key role in insulin signalling, no significant associations were observed. However, in the complete sample a trend towards association with fasting glucose levels was observed ($p$=0.05), but because we had to correct for multiple testing this association was considered non-significant

A limitation of this study is the small sample size. With a power of 80%, a significance threshold of 0.05 and a group-size ratio (e.g. $n_1$:$n_2$) of 4:1, 2:1 and 1:1, we are able to detect differences between two means with effect sizes (d) of approximately 0.29, 0.24 and 0.23 in the complete sample, and with effect sizes of 0.43, 0.36 and 0.34 in the men-only sample (which represents the smallest group). Since an effect size of 0.20 represents a small effect, this study has low power to identify small differences and consequently the false-negative rate is high. Another limitation is the large number of tests performed. Although we attempt to control for multiple testing (see methods) this may not be sufficient, but applying a more stringent correction method will increase the number of false-negatives. Since some results might reflect a random association by chance, we provided for each SNP a detailed literature overview (Table S5-S15). Results consistent with previous studies are likely to represent a true association. In contrast, results in conflict with the literature may reflect false-positive findings, and should be interpreted cautiously.

In conclusion, this study showed that in young adults common SNPs in *ENPP1* (K121Q)*, FAAH* (P129T), *GAD2* (-243A>G), *IL6* (-174G>C), *PPARγ* (P12A), *PYY* (R72T) and *UCP2* (-

866G>A) are already associated with metabolic risk factors, although some associations are in disagreement with the literature. Most SNPs acted sex-specific, and if confirmed in other populations these sex-specific associations may be important in the development of future genotype-based medicine.

## 4.6 ACKNOWLEDGMENTS

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## 4.8 SUPPLEMENTARY MATERIAL I

**Table S1.** Covariate(s) incorporated into the models for the complete sample and for men and women separately.

| | Covariate(s) | | |
|---|---|---|---|
| Trait | All | Men only | Women only |
| Birth weight | Sex, gestational age and chorionicity | Gestational age and chorionicity | Gestational age |
| Body mass | Age and body height | Age and body height | Age and body height |
| BMI[a] | Age | Age | Age |
| Fat mass[a] | Sex, age and body height | Age | Age |
| Lean body mass | Sex, age and body height | Age and body height | Age and body height |
| S4SF[a] | Zygosity, sex and age | Age | Zygosity and age |
| WHR | Zygosity, sex and age | Age | Zygosity and age |
| IGFBP1[a] | Zygosity, sex and BMI | Zygosity and BMI | BMI |
| Fasting glucose | Zygosity, sex and WHR | Zygosity and WHR | WHR |
| Fasting insulin[a] | Age and S4SF | Age and S4SF | Age and S4SF |
| Leptin[a] | Sex, age and S4SF | Age and S4SF | Age and S4SF |
| Total cholesterol[b] | Zygosity, age and S4SF | Age and S4SF | Age and S4SF |
| LDL-cholesterol | Sex, age and S4SF | Age and S4SF | Age and S4SF |
| HDL-cholesterol[c] | Sex and WHR | WHR | WHR |
| Triglycerides[a] | Sex and S4SF | S4SF | S4SF |
| NEFA[d] | Sex and S4SF | - | S4SF |

[a]Transformed to natural logarithmic scale. [b]Transformed to natural logarithmic scale in the women-only sample. [c]Transformed to natural logarithmic scale in the men-only and women-only sample. [d]Transformed to natural logarithmic scale in the complete and men-only sample.

**Table S2.** Factor loadings of the pre-diabetic phenotypes.

| | Factors | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | F | α |
| Birth weight | -0.03 | -0.05 | 0.14 | 0.13 | 0.19 | -0.67 | - | 0.05 |
| Body mass | **0.97** | 0.00 | -0.03 | 0.05 | -0.03 | -0.12 | 1 | 0.01 |
| BMI | **0.96** | -0.01 | -0.04 | 0.05 | -0.01 | -0.12 | 1 | 0.01 |
| Fat mass | **0.85** | 0.02 | 0.10 | 0.11 | 0.11 | 0.05 | 1 | 0.01 |
| Lean body mass | **0.80** | 0.00 | -0.13 | 0.03 | -0.09 | -0.26 | 1 | 0.01 |
| S4SF | **0.87** | -0.04 | 0.01 | -0.09 | 0.01 | 0.11 | 1 | 0.01 |
| WHR | 0.66 | 0.08 | 0.01 | 0.05 | -0.03 | 0.21 | - | 0.05 |
| IGFBP1 | -0.01 | -0.06 | **0.80** | -0.01 | 0.06 | 0.08 | 3 | 0.025 |
| Fasting glucose | 0.02 | -0.08 | **-0.74** | 0.16 | 0.08 | 0.08 | 3 | 0.025 |
| Fasting insulin | 0.07 | 0.02 | -0.23 | **0.78** | -0.13 | 0.17 | 4 | 0.025 |
| Leptin | 0.06 | -0.01 | 0.02 | **0.78** | 0.10 | -0.22 | 4 | 0.025 |
| Total cholesterol | 0.01 | **0.97** | 0.06 | 0.06 | 0.20 | 0.03 | 2 | 0.025 |
| LDL-cholesterol | 0.03 | **0.95** | -0.03 | -0.04 | -0.05 | -0.04 | 2 | 0.025 |
| HDL-cholesterol | -0.02 | 0.23 | 0.06 | 0.06 | **0.88** | 0.09 | 5 | 0.05 |
| Triglycerides | -0.02 | 0.33 | 0.36 | 0.33 | -0.57 | 0.23 | - | 0.05 |
| NEFA | -0.07 | -0.07 | 0.17 | 0.09 | 0.25 | 0.70 | - | 0.05 |
| Total variance (%) | 27.6 | 12.7 | 9.1 | 8.8 | 8.1 | 7.9 | - | - |
| Cumulative variance (%) | 27.6 | 40.3 | 49.4 | 58.2 | 66.3 | 74.2 | - | - |

Factor loadings >0.70 are shown in **boldface type**. α = significance threshold, BMI = body mass index, F = factor, IGFBP1 = insulin-like growth factor protein 1, NEFA = non-esterified fatty acids, S4SF = sum of four skinfolds, WHR = waist-to-hip ratio.

**Table S3.** Genotype frequencies and numbers, and allele frequencies of the SNPs calculated using a sub-sample, that only included one randomly selected twin per pair.

| | | Genotype | | | Alelle | |
|---|---|---|---|---|---|---|
| | | Homozygous major allele (n) | Heterozygous (n) | Homozygous minor allele (n) | Major allele | Minor allele |
| *PPARγ* | P12A | 0.794 (247) | 0.199 (62) | 0.007 (2) | 0.894 | 0.106 |
| *IL6* | -174 G>C | 0.340 (102) | 0.470 (141) | 0.190 (57) | 0.575 | 0.425 |
| *GAD2* | -243 A>G | 0.672 (209) | 0.293 (91) | 0.035 (11) | 0.818 | 0.182 |
| *PYY* | R72T | 0.407 (126) | 0.474 (147) | 0.119 (37) | 0.644 | 0.356 |
| *ENPP1* | K121Q | 0.800 (252) | 0.190 (60) | 0.01 (3) | 0.895 | 0.105 |
| *UCP2* | -866 G>A | 0.432 (133) | 0.467 (144) | 0.101 (31) | 0.666 | 0.334 |
| *FAAH* | P129T | 0.673 (206) | 0.301 (92) | 0.026 (8) | 0.824 | 0.176 |
| *KCNJ11* | E23K | 0.395 (120) | 0.467 (142) | 0.138 (42) | 0.628 | 0.372 |
| *AGTR1* | 1166 A>C | 0.476 (147) | 0.450 (139) | 0.074 (23) | 0.701 | 0.299 |
| *CYP1A1* | 6235 T>C | 0.851 (262) | 0.143 (44) | 0.006 (2) | 0.922 | 0.078 |
| *IRS2* | G1057D | 0.407 (123) | 0.480 (145) | 0.113 (34) | 0.647 | 0.353 |

**Table S4.** Association between T2D related metabolic risk factors and SNPs in the *AGTR1*, *CYP1A1*, *ENPP1*, *FAAH*, *GAD2*, *IL6*, *IRS2*, *KCNJ11*, *PPARγ*, *PYY* and the *UCP2* gene adjusted for significant covariates carried out on the complete, men-only and women-only samples.

| | Complete sample *AGTR1* (1166A>C) | | | |
|---|---|---|---|---|
| Trait | AA | AC | CC | $p_G$ |
| Birth weight | 2555 (2505-2606) | 2544 (2491-2596) | 2500 (2383-2618) | 0.69 |
| Body mass | 64.5 (63.3-65.7) | 64.6 (63.4-65.9) | 66.7 (63.9-69.5) | 0.35 |
| BMI[a] | 21.7 (21.3-22.1) | 21.7 (21.3-22.1) | 22.5 (21.6-23.5) | 0.29 |
| Fat mass[a] | 13.7 (13.1-14.3) | 14.4 (13.7-15.1) | 14.3 (12.8-16.0) | 0.30 |
| Lean body mass | 49.7 (49.0-50.4) | 49.5 (48.8-50.2) | 51.3 (49.7-52.9) | 0.10 |
| S4SF[a] | 42.7 (40.5-44.9) | 44.3 (42.1-46.7) | 46.1 (40.9-52.0) | 0.37 |
| WHR | 77.8 (77.1-78.4) | 77.4 (76.8-78.1) | 78.2 (76.7-79.7) | 0.52 |
| IGFBP1[a] | 15.1 (13.9-16.5) | 13.4 (12.3-14.7) | 14.5 (11.8-18.0) | 0.15 |
| Fasting glucose | 4.71 (4.66-4.76) | 4.73 (4.67-4.78) | 4.70 (4.57-4.83) | 0.85 |
| Fasting insulin[a] | 35.3 (33.4-37.2) | 35.7 (33.7-37.8) | 36.6 (32.1-41.8) | 0.86 |
| Leptin[a] | 4.40 (4.07-4.76) | 4.36 (4.02-4.72) | 4.49 (3.73-5.41) | 0.95 |
| Total cholesterol | 5.03 (4.90-5.16) | 5.01 (4.88-5.14) | 5.08 (4.79-5.37) | 0.89 |
| LDL-cholesterol | 2.96 (2.84-3.07) | 2.97 (2.86-3.09) | 3.03 (2.77-3.29) | 0.88 |
| HDL-cholesterol | 1.62 (1.57-1.67) | 1.61 (1.55-1.66) | 1.57 (1.45-1.69) | 0.72 |
| Triglycerides[a] | 0.89 (0.85-0.95) | 0.85 (0.81-0.91) | 0.86 (0.75-0.98) | 0.51 |
| NEFA[a] | 0.55 (0.53-0.58) | 0.57 (0.54-0.60) | 0.53 (0.47-0.60) | 0.56 |

[a]Geometric least squares mean (95% confidence interval). $p_G$ = *p*-value general association model.

| | Men only sample *AGTR1* (1166A>C) | | | |
|---|---|---|---|---|
| Trait | AA | AC | CC | $p_G$ |
| Birth weight | 2656 (2581-2732) | 2559 (2484-2633) | 2651 (2487-2814) | 0.15 |
| Body mass | 69.1 (67.5-70.8) | 69.0 (67.4-70.6) | 72.3 (68.6-76.0) | 0.26 |
| BMI[a] | 21.6 (21.1-22.2) | 21.7 (21.2-22.2) | 22.8 (21.5-24.0) | 0.24 |
| Fat mass[a] | 11.3 (10.4-12.2) | 12.4 (11.6-13.4) | 12.3 (10.3-14.7) | 0.16 |
| Lean body mass | 57.1 (56.0-58.1) | 56.2 (55.2-57.3) | 59.6 (57.1-62.0) | 0.04 |
| S4SF[a] | 32.7 (30.1-35.6) | 36.2 (33.5-39.3) | 35.8 (29.6-43.2) | 0.19 |
| WHR | 82.8 (81.8-83.8) | 82.5 (81.5-83.4) | 83.6 (81.4-85.9) | 0.61 |
| IGFBP1[a] | 12.0 (10.8-13.3) | 11.6 (10.5-12.8) | 13.0 (10.2-16.5) | 0.66 |
| Fasting glucose | 4.88 (4.79-4.97) | 4.87 (4.79-4.96) | 4.77 (4.56-4.97) | 0.62 |
| Fasting insulin[a] | 31.9 (29.3-34.7) | 32.4 (29.9-35.2) | 32.2 (26.7-38.8) | 0.97 |
| Leptin[a] | 1.62 (1.41-1.85) | 1.48 (1.30-1.68) | 1.73 (1.28-2.35) | 0.48 |
| Total cholesterol | 4.96 (4.77-5.15) | 4.74 (4.56-4.93) | 4.95 (4.52-5.39) | 0.22 |
| LDL-cholesterol | 3.11 (2.93-3.29) | 2.92 (2.74-3.10) | 3.30 (2.89-3.72) | 0.14 |
| HDL-cholesterol[a] | 1.36 (1.29-1.43) | 1.37 (1.30-1.44) | 1.23 (1.10-1.38) | 0.23 |
| Triglycerides[a] | 0.89 (0.83-0.97) | 0.78 (0.73-0.85) | 0.85 (0.72-1.01) | 0.06 |
| NEFA[a] | 0.46 (0.42-0.50) | 0.51 (0.47-0.55) | 0.45 (0.38-0.54) | 0.12 |

[a]Geometric least squares mean (95% confidence interval). $p_G$ = *p*-value general association model.

| | Women only sample *AGTR1* (1166A>C) | | | |
|---|---|---|---|---|
| Trait | AA | AC | CC | $p_G$ |
| Birth weight | 2460 (2393-2527) | 2518 (2448-2588) | 2356 (2191-2522) | 0.15 |
| Body mass | 60.5 (58.8-62.1) | 60.4 (58.7-62.2) | 60.0 (56.0-64.1) | 0.98 |
| BMI[a] | 21.7 (21.2-22.3) | 21.8 (21.2-22.5) | 21.8 (20.5-23.3) | 0.96 |
| Fat mass[a] | 16.9 (16.1-17.8) | 16.3 (15.5-17.2) | 16.8 (14.9-19.0) | 0.57 |
| Lean body mass | 42.9 (42.0-43.7) | 43.3 (42.4-44.2) | 42.9 (40.9-45.0) | 0.76 |
| S4SF[a] | 55.3 (51.9-58.8) | 53.8 (50.3-57.5) | 57.1 (48.9-66.7) | 0.71 |
| WHR | 72.7 (71.9-73.5) | 72.4 (71.6-73.3) | 72.7 (70.7-74.7) | 0.89 |
| IGFBP1[a] | 18.5 (16.3-20.9) | 15.8 (13.7-18.1) | 16.5 (11.7-23.2) | 0.24 |
| Fasting glucose | 4.53 (4.47-4.60) | 4.58 (4.51-4.65) | 4.58 (4.41-4.74) | 0.62 |
| Fasting insulin[a] | 39.2 (36.6-42.0) | 39.7 (36.8-42.7) | 42.4 (35.4-50.8) | 0.73 |
| Leptin[a] | 12.2 (11.2-13.3) | 12.0 (10.9-13.1) | 11.5 (9.25-14.2) | 0.84 |
| Total cholesterol[a] | 5.03 (4.88-5.19) | 5.14 (4.98-5.32) | 4.98 (4.61-5.38) | 0.54 |
| LDL-cholesterol | 2.81 (2.68-2.95) | 2.98 (2.83-3.12) | 2.83 (2.50-3.17) | 0.22 |
| HDL-cholesterol[a] | 1.80 (1.73-1.87) | 1.76 (1.68-1.83) | 1.83 (1.66-2.03) | 0.62 |
| Triglycerides[a] | 0.90 (0.84-0.97) | 0.90 (0.83-0.98) | 0.88 (0.72-1.07) | 0.96 |
| NEFA | 0.70 (0.66-0.74) | 0.67 (0.63-0.71) | 0.69 (0.59-0.80) | 0.62 |

[a]Geometric least squares mean (95% confidence interval). $p_G$ = *p*-value general association model.

| | Complete sample *CYP1A1* (6235T>C) | | | |
|---|---|---|---|---|
| Trait | TT | TC | CC | $p_G$[b] |
| Birth weight | 2544 (2504-2584) | 2548 (2458-2639) | 2747 (2368-3126) | 0.90 |
| Body mass | 64.2 (63.3-65.1) | 66.5 (64.4-68.6) | 67.9 (59.4-76.3) | 0.05 |
| BMI[a] | 21.6 (21.3-21.9) | 22.4 (21.7-23.1) | 22.8 (20.1-25.8) | 0.04 |
| Fat mass[a] | 13.8 (13.3-14.3) | 14.7 (13.5-16.0) | 17.0 (12.1-23.8) | 0.15 |
| Lean body mass | 49.5 (49.0-50.0) | 50.7 (49.5-51.9) | 50.4 (45.7-55.1) | 0.06 |
| S4SF[a] | 42.6 (41.0-44.3) | 46.0 (42.1-50.4) | 50.2 (35.4-71.4) | 0.10 |
| WHR | 77.5 (77.0-77.9) | 78.3 (77.2-79.4) | 80.6 (76.2-85.0) | 0.14 |
| IGFBP1[a] | 14.4 (13.5-15.4) | 14.6 (12.4-17.1) | 10.1 (5.09-20.1) | 0.90 |
| Fasting glucose | 4.73 (4.69-4.77) | 4.73 (4.64-4.83) | 4.90 (4.43-5.36) | 0.92 |
| Fasting insulin[a] | 35.2 (33.8-36.7) | 38.1 (34.5-42.0) | 52.1 (34.4-78.8) | 0.16 |
| Leptin[a] | 4.31 (4.06-4.57) | 4.79 (4.15-5.52) | 3.95 (2.23-6.99) | 0.17 |
| Total cholesterol | 5.04 (4.94-5.14) | 4.91 (4.68-5.13) | 5.43 (4.54-6.32) | 0.30 |
| LDL-cholesterol | 2.98 (2.89-3.07) | 2.86 (2.66-3.06) | 3.52 (2.74-4.30) | 0.33 |
| HDL-cholesterol | 1.61 (1.57-1.65) | 1.63 (1.54-1.73) | 1.46 (1.09-1.82) | 0.61 |
| Triglycerides[a] | 0.87 (0.83-0.91) | 0.84 (0.76-0.93) | 1.78 (1.18-2.69) | 0.61 |
| NEFA[a] | 0.55 (0.53-0.57) | 0.58 (0.53-0.63) | 0.67 (0.46-0.98) | 0.38 |

[a]Geometric least squares mean (95% confidence interval). [b]Homozygous major allele *vs* heterozygous only (homozygous minor allele group not included, because it contains less then 10 individuals). $p_G$ = *p*-value general association model.

| | Men only sample *CYP1A1* (6235T>C) | | | |
|---|---|---|---|---|
| Trait | TT | TC | CC | $p_G$[b] |
| Birth weight | 2592 (2535-2649) | 2659 (2515-2804) | 2621 (1883-3360) | 0.38 |
| Body mass | 68.8 (67.6-70.0) | 72.0 (68.9-75.2) | 71.3 (56.2-86.5) | 0.06 |
| BMI[a] | 21.6 (21.2-22.0) | 22.9 (21.9-24.0) | 22.3 (17.7-28.1) | 0.02 |
| Fat mass[a] | 11.7 (11.1-12.4) | 12.7 (11.0-14.7) | 16.0 (8.35-30.8) | 0.31 |
| Lean body mass | 56.6 (55.8-57.4) | 58.5 (56.5-60.6) | 53.5 (42.9-64.0) | 0.08 |
| S4SF[a] | 34.0 (32.0-36.1) | 37.5 (32.1-43.8) | 52.9 (25.5-110) | 0.23 |
| WHR | 82.5 (81.8-83.2) | 83.7 (81.9-85.6) | 85.2 (76.3-94.1) | 0.22 |
| IGFBP1[a] | 11.8 (11.0-12.7) | 12.1 (9.89-14.9) | 16.2 (6.15-42.5) | 0.82 |
| Fasting glucose | 4.86 (4.80-4.93) | 4.96 (4.80-5.13) | 3.95 (3.20-4.71) | 0.26 |
| Fasting insulin[a] | 32.1 (30.3-34.1) | 33.3 (28.4-39.0) | 42.6 (18.8-96.8) | 0.69 |
| Leptin[a] | 1.53 (1.39-1.69) | 1.95 (1.50-2.52) | 1.25 (0.37-4.29) | 0.09 |
| Total cholesterol | 4.89 (4.75-5.04) | 4.61 (4.25-4.98) | 6.03 (4.27-7.78) | 0.15 |
| LDL-cholesterol | 3.08 (2.95-3.21) | 2.79 (2.44-3.14) | 4.76 (3.05-6.46) | 0.13 |
| HDL-cholesterol[a] | 1.34 (1.29-1.39) | 1.41 (1.28-1.55) | 1.37 (0.86-2.18) | 0.30 |
| Triglycerides[a] | 0.85 (0.80-0.90) | 0.74 (0.64-0.86) | 2.74 (1.21-6.20) | 0.08 |
| NEFA[a] | 0.47 (0.45-0.50) | 0.53 (0.46-0.62) | 0.98 (0.48-2.02) | 0.17 |

[a]Geometric least squares mean (95% confidence interval). [b]Homozygous major allele *vs* heterozygous only (homozygous minor allele group not included, because it contains less then 10 individuals). $p_G$ = *p*-value general association model.

| | Women only sample *CYP1A1* (6235T>C) | | | |
|---|---|---|---|---|
| Trait | TT | TC | CC | $p_G$[b] |
| Birth weight | 2488 (2435-2541) | 2479 (2363-2596) | 2630 (2192-3067) | 0.92 |
| Body mass | 59.9 (58.7-61.2) | 61.6 (58.8-64.4) | 63.5 (52.5-74.5) | 0.28 |
| BMI[a] | 21.6 (21.2-22.0) | 22.1 (21.1-23.1) | 22.7 (19.0-27.0) | 0.36 |
| Fat mass[a] | 16.4 (15.8-17.1) | 17.0 (15.6-18.5) | 17.6 (12.9-24.0) | 0.47 |
| Lean body mass | 42.8 (42.2-43.5) | 43.9 (42.5-45.3) | 45.4 (40.1-50.8) | 0.16 |
| S4SF[a] | 53.3 (50.8-56.0) | 57.0 (51.2-63.4) | 52.1 (34.9-77.8) | 0.26 |
| WHR | 72.5 (71.9-73.1) | 73.1 (71.8-74.5) | 74.9 (70.2-79.7) | 0.38 |
| IGFBP1[a] | 17.5 (15.8-19.3) | 18.6 (14.7-23.4) | 9.12 (3.72-22.3) | 0.67 |
| Fasting glucose | 4.57 (4.52-4.63) | 4.55 (4.44-4.67) | 4.78 (4.36-5.19) | 0.73 |
| Fasting insulin[a] | 39.1 (37.0-41.3) | 41.5 (36.7-46.9) | 68.0 (42.1-110) | 0.38 |
| Leptin[a] | 11.8 (11.1-12.6) | 12.6 (10.9-14.7) | 8.81 (4.93-15.7) | 0.41 |
| Total cholesterol[a] | 5.06 (4.94-5.19) | 5.06 (4.79-5.34) | 4.96 (4.06-6.08) | 0.95 |
| LDL-cholesterol | 2.86 (2.75-2.97) | 2.91 (2.67-3.15) | 2.85 (2.01-3.69) | 0.73 |
| HDL-cholesterol[a] | 1.80 (1.74-1.86) | 1.77 (1.65-1.90) | 1.45 (1.10-1.90) | 0.68 |
| Triglycerides[a] | 0.88 (0.83-0.93) | 0.95 (0.83-1.08) | 1.54 (0.92-2.56) | 0.30 |
| NEFA | 0.68 (0.65-0.71) | 0.69 (0.62-0.76) | 0.67 (0.38-0.95) | 0.78 |

[a]Geometric least squares mean (95% confidence interval). [b]Homozygous major allele *vs* heterozygous only (homozygous minor allele group not included, because it contains less then 10 individuals). $p_G$ = *p*-value general association model.

| | Complete sample *ENPP1* (K121Q) | | | |
|---|---|---|---|---|
| Trait | KK | KQ | QQ | $p_G$[b] |
| Birth weight | 2540 (2499-2580) | 2573 (2495-2651) | 2644 (2289-2999) | 0.45 |
| Body mass | 64.2 (63.3-65.2) | 65.0 (63.2-66.8) | 63.7 (55.6-71.8) | 0.46 |
| BMI[a] | 21.6 (21.3-21.9) | 21.9 (21.3-22.5) | 21.7 (19.2-24.5) | 0.43 |
| Fat mass[a] | 13.9 (13.4-14.4) | 13.6 (12.7-14.7) | 15.0 (10.9-20.6) | 0.61 |
| Lean body mass | 49.4 (48.9-49.9) | 50.2 (49.2-51.2) | 48.2 (43.6-52.8) | 0.17 |
| S4SF[a] | 43.0 (41.3-44.7) | 42.7 (39.4-46.2) | 53.2 (37.6-75.3) | 0.89 |
| WHR | 77.5 (77.0-78.0) | 77.4 (76.5-78.4) | 81.4 (77.2-85.6) | 0.91 |
| IGFBP1[a] | 14.5 (13.5-15.5) | 14.4 (12.6-16.5) | 7.97 (3.89-16.3) | 0.93 |
| Fasting glucose | 4.72 (4.68-4.76) | 4.78 (4.69-4.86) | 4.52 (4.16-4.88) | 0.18 |
| Fasting insulin[a] | 35.6 (34.1-37.1) | 35.5 (32.7-38.6) | 42.2 (28.6-62.1) | 0.98 |
| Leptin[a] | 4.33 (4.08-4.60) | 4.46 (3.95-5.04) | 3.42 (1.99-5.87) | 0.65 |
| Total cholesterol | 5.03 (4.93-5.13) | 4.96 (4.77-5.15) | 5.01 (4.13-5.90) | 0.53 |
| LDL-cholesterol | 2.99 (2.90-3.08) | 2.84 (2.66-3.01) | 2.54 (1.73-3.34) | 0.09 |
| HDL-cholesterol | 1.60 (1.56-1.64) | 1.68 (1.60-1.76) | 1.38 (0.92-1.83) | 0.10 |
| Triglycerides[a] | 0.86 (0.82-0.90) | 0.88 (0.81-0.96) | 1.26 (0.84-1.88) | 0.66 |
| NEFA[a] | 0.56 (0.54-0.58) | 0.54 (0.50-0.58) | 0.82 (0.58-1.15) | 0.49 |

[a]Geometric least squares mean (95% confidence interval). [b]Homozygous major allele *vs* heterozygous only (homozygous minor allele group not included, because it contains less then 10 individuals). $p_G$ = *p*-value general association model.

| | Men only sample *ENPP1* (K121Q) | | | |
|---|---|---|---|---|
| Trait | KK | KQ | QQ | $p_G$[b] |
| Birth weight | 2601 (2543-2660) | 2621 (2501-2741) | 2550 (2036-3064) | 0.76 |
| Body mass | 68.8 (67.6-70.1) | 70.8 (68.2-73.4) | 67.7 (57.9-77.4) | 0.18 |
| BMI[a] | 21.6 (21.2-22.0) | 22.1 (21.3-23.0) | 21.4 (18.5-24.7) | 0.23 |
| Fat mass[a] | 11.8 (11.1-12.5) | 11.6 (10.2-13.2) | 11.7 (7.76-17.7) | 0.85 |
| Lean body mass | 56.5 (55.7-57.3) | 58.6 (56.9-60.2) | 55.1 (48.6-61.6) | 0.03 |
| S4SF[a] | 33.7 (31.7-35.8) | 36.9 (32.4-42.1) | 39.5 (25.7-60.9) | 0.18 |
| WHR | 82.5 (81.8-83.3) | 82.5 (80.9-84.0) | 83.4 (78.0-88.8) | 0.89 |
| IGFBP1[a] | 12.2 (11.3-13.1) | 10.9 (9.17-12.9) | 15.9 (6.09-41.6) | 0.24 |
| Fasting glucose | 4.86 (4.79-4.93) | 4.94 (4.80-5.09) | 4.61 (4.13-5.09) | 0.25 |
| Fasting insulin[a] | 32.0 (30.1-34.0) | 33.1 (29.1-37.7) | 41.1 (23.2-72.8) | 0.63 |
| Leptin[a] | 1.55 (1.41-1.72) | 1.52 (1.22-1.88) | 1.05 (0.45-2.45) | 0.84 |
| Total cholesterol | 4.84 (4.70-4.98) | 4.80 (4.49-5.10) | 7.11 (5.45-8.76) | 0.81 |
| LDL-cholesterol | 3.03 (2.90-3.17) | 2.96 (2.67-3.26) | 3.20 (2.09-4.30) | 0.48 |
| HDL-cholesterol[a] | 1.35 (1.30-1.41) | 1.37 (1.26-1.48) | 1.28 (0.78-2.10) | 0.78 |
| Triglycerides[a] | 0.82 (0.78-0.87) | 0.83 (0.73-0.93) | 1.21 (0.71-2.05) | 0.97 |
| NEFA[a] | 0.49 (0.46-0.52) | 0.41 (0.36-0.47) | 0.86 (0.53-1.41) | 0.02 |

[a]Geometric least squares mean (95% confidence interval). [b]Homozygous major allele *vs* heterozygous only (homozygous minor allele group not included, because it contains less then 10 individuals). $p_G$ = *p*-value general association model.

| | Women only sample *ENPP1* (K121Q) | | | |
|---|---|---|---|---|
| Trait | KK | KQ | QQ | $p_G$[b] |
| Birth weight | 2476 (2423-2530) | 2512 (2406-2618) | 2648 (2195-3101) | 0.55 |
| Body mass | 60.1 (58.9-61.4) | 60.3 (57.9-62.7) | 60.7 (49.4-71.9) | 0.92 |
| BMI[a] | 21.7 (21.3-22.1) | 21.7 (20.9-22.5) | 22.0 (18.4-26.3) | 0.97 |
| Fat mass[a] | 16.5 (15.9-17.2) | 16.7 (15.5-18.0) | 18.0 (12.8-25.5) | 0.75 |
| Lean body mass | 43.0 (42.3-43.6) | 42.7 (41.5-44.0) | 42.6 (36.9-48.3) | 0.76 |
| S4SF[a] | 54.4 (51.8-57.1) | 51.5 (46.9-56.5) | 67.4 (43.7-104) | 0.30 |
| WHR | 72.4 (71.8-73.0) | 72.7 (71.5-73.8) | 79.1 (73.7-84.5) | 0.73 |
| IGFBP1[a] | 17.3 (15.6-19.1) | 18.6 (15.4-22.6) | 7.14 (2.91-17.5) | 0.49 |
| Fasting glucose | 4.57 (4.52-4.62) | 4.57 (4.47-4.67) | 4.44 (3.98-4.90) | 0.97 |
| Fasting insulin[a] | 39.7 (37.5-41.9) | 38.5 (34.6-42.8) | 43.3 (26.3-71.4) | 0.64 |
| Leptin[a] | 11.7 (10.9-12.5) | 12.2 (10.8-13.9) | 11.2 (6.20-20.3) | 0.52 |
| Total cholesterol[a] | 5.09 (4.97-5.22) | 5.04 (4.81-5.29) | 4.36 (3.51-5.43) | 0.72 |
| LDL-cholesterol | 2.92 (2.81-3.04) | 2.73 (2.52-2.95) | 2.55 (1.56-3.53) | 0.12 |
| HDL-cholesterol[a] | 1.77 (1.71-1.83) | 1.89 (1.78-2.01) | 1.39 (1.05-1.84) | 0.05 |
| Triglycerides[a] | 0.89 (0.83-0.94) | 0.92 (0.82-1.03) | 1.45 (0.85-2.48) | 0.57 |
| NEFA | 0.67 (0.64-0.70) | 0.72 (0.66-0.78) | 0.81 (0.53-1.09) | 0.17 |

[a]Geometric least squares mean (95% confidence interval). [b]Homozygous major allele *vs* heterozygous only (homozygous minor allele group not included, because it contains less then 10 individuals). $p_G$ = *p*-value general association model.

| | Complete sample *FAAH* (P129T) | | | |
|---|---|---|---|---|
| Trait | PP | PT | TT | $p_G$ |
| Birth weight | 2522 (2477-2567) | 2573 (2512-2635) | 2706 (2514-2898) | 0.10 |
| Body mass | 64.0 (63.0-65.0) | 65.0 (63.6-66.4) | 64.5 (60.1-68.9) | 0.53 |
| BMI[a] | 21.6 (21.2-21.9) | 22.0 (21.5-22.4) | 21.7 (20.3-23.2) | 0.35 |
| Fat mass[a] | 13.9 (13.4-14.5) | 13.8 (13.1-14.6) | 13.2 (11.1-15.7) | 0.82 |
| Lean body mass | 49.3 (48.7-49.8) | 50.2 (49.4-51.0) | 50.4 (47.9-52.9) | 0.15 |
| S4SF[a] | 43.4 (41.6-45.4) | 42.8 (40.3-45.4) | 40.9 (34.0-49.3) | 0.79 |
| WHR | 77.3 (76.8-77.9) | 78.0 (77.2-78.7) | 77.2 (75.0-79.5) | 0.37 |
| IGFBP1[a] | 13.9 (12.9-15.0) | 15.4 (13.9-17.1) | 13.0 (9.24-18.2) | 0.26 |
| Fasting glucose | 4.72 (4.67-4.77) | 4.73 (4.67-4.79) | 4.80 (4.59-5.00) | 0.76 |
| Fasting insulin[a] | 35.5 (33.9-37.2) | 36.2 (33.9-38.6) | 35.3 (28.5-43.6) | 0.89 |
| Leptin[a] | 4.36 (4.07-4.67) | 4.40 (4.00-4.84) | 3.45 (2.56-4.64) | 0.29 |
| Total cholesterol | 4.99 (4.88-5.10) | 5.03 (4.88-5.17) | 5.32 (4.85-5.78) | 0.40 |
| LDL-cholesterol | 2.93 (2.83-3.03) | 3.04 (2.90-3.17) | 3.05 (2.63-3.46) | 0.39 |
| HDL-cholesterol | 1.63 (1.59-1.68) | 1.57 (1.51-1.64) | 1.69 (1.49-1.88) | 0.21 |
| Triglycerides[a] | 0.86 (0.82-0.90) | 0.87 (0.81-0.93) | 1.02 (0.82-1.26) | 0.29 |
| NEFA[a] | 0.56 (0.54-0.58) | 0.55 (0.52-0.58) | 0.55 (0.45-0.67) | 0.84 |

[a]Geometric least squares mean (95% confidence interval). $p_G$ = *p*-value general association model.

| Trait | Men only sample *FAAH* (P129T) PP | PT | TT | $p_G$ |
|---|---|---|---|---|
| Birth weight | 2558 (2492-2625) | 2626 (2538-2715) | 2872 (2630-3115) | 0.03 |
| Body mass | 68.6 (67.2-70.0) | 69.5 (67.7-71.4) | 69.8 (64.8-74.8) | 0.67 |
| BMI[a] | 21.6 (21.1-22.0) | 21.8 (21.2-22.4) | 21.9 (20.3-23.6) | 0.78 |
| Fat mass[a] | 11.9 (11.2-12.7) | 11.3 (10.4-12.3) | 11.1 (8.87-13.9) | 0.55 |
| Lean body mass | 56.4 (55.5-57.4) | 57.2 (56.0-58.5) | 57.9 (54.5-61.3) | 0.49 |
| S4SF[a] | 34.7 (32.3-37.2) | 33.2 (30.3-36.5) | 31.1 (24.4-39.7) | 0.61 |
| WHR | 82.4 (81.5-83.2) | 82.5 (81.4-83.6) | 83.6 (80.6-86.6) | 0.73 |
| IGFBP1[a] | 11.9 (10.9-13.1) | 12.3 (10.9-13.9) | 10.9 (7.83-15.2) | 0.78 |
| Fasting glucose | 4.84 (4.77-4.92) | 4.90 (4.80-5.00) | 5.03 (4.74-5.33) | 0.38 |
| Fasting insulin[a] | 31.4 (29.3-33.7) | 33.3 (30.3-36.7) | 37.2 (28.4-48.6) | 0.35 |
| Leptin[a] | 1.55 (1.38-1.74) | 1.55 (1.33-1.82) | 1.18 (0.77-1.83) | 0.49 |
| Total cholesterol | 4.76 (4.60-4.92) | 4.89 (4.68-5.11) | 5.17 (4.59-5.75) | 0.32 |
| LDL-cholesterol | 2.98 (2.82-3.14) | 3.06 (2.85-3.27) | 3.11 (2.53-3.68) | 0.78 |
| HDL-cholesterol[a] | 1.36 (1.30-1.42) | 1.33 (1.26-1.42) | 1.48 (1.26-1.73) | 0.46 |
| Triglycerides[a] | 0.82 (0.76-0.87) | 0.81 (0.74-0.88) | 1.10 (0.86-1.41) | 0.06 |
| NEFA[a] | 0.47 (0.44-0.51) | 0.48 (0.44-0.53) | 0.51 (0.40-0.66) | 0.82 |

[a]Geometric least squares mean (95% confidence interval). $p_G$ = *p*-value general association model.

| Trait | Women only sample *FAAH* (P129T) PP | PT | TT | $p_G$[b] |
|---|---|---|---|---|
| Birth weight | 2478 (2421-2536) | 2517 (2433-2601) | 2430 (2128-2733) | 0.44 |
| Body mass | 60.0 (58.6-61.4) | 61.1 (59.1-63.2) | 59.9 (52.1-67.8) | 0.34 |
| BMI[a] | 21.6 (21.1-22.0) | 22.1 (21.4-22.8) | 21.4 (18.9-24.2) | 0.21 |
| Fat mass[a] | 16.5 (15.8-17.2) | 16.8 (15.8-17.8) | 16.7 (13.5-20.7) | 0.60 |
| Lean body mass | 42.7 (42.0-43.4) | 43.8 (42.8-44.8) | 43.4 (39.5-47.3) | 0.08 |
| S4SF[a] | 54.5 (51.7-57.5) | 54.4 (50.4-58.7) | 55.9 (42.3-73.8) | 0.93 |
| WHR | 72.5 (71.8-73.1) | 73.2 (72.3-74.2) | 69.5 (66.2-72.9) | 0.19 |
| IGFBP1[a] | 16.7 (15.0-18.6) | 19.2 (16.3-22.6) | 15.0 (8.00-28.3) | 0.17 |
| Fasting glucose | 4.58 (4.53-4.64) | 4.53 (4.45-4.61) | 4.49 (4.19-4.79) | 0.30 |
| Fasting insulin[a] | 39.9 (37.6-42.4) | 39.0 (35.7-42.6) | 32.5 (22.8-46.3) | 0.44 |
| Leptin[a] | 12.0 (11.1-12.9) | 11.6 (10.4-12.9) | 10.1 (6.75-15.2) | 0.56 |
| Total cholesterol[a] | 5.08 (4.95-5.22) | 5.09 (4.90-5.29) | 5.52 (4.78-6.36) | 0.92 |
| LDL-cholesterol | 2.86 (2.74-2.98) | 2.99 (2.82-3.17) | 2.98 (2.39-3.56) | 0.20 |
| HDL-cholesterol[a] | 1.82 (1.76-1.88) | 1.73 (1.64-1.81) | 1.72 (1.42-2.08) | 0.07 |
| Triglycerides[a] | 0.89 (0.83-0.95) | 0.92 (0.84-1.01) | 0.89 (0.62-1.29) | 0.42 |
| NEFA | 0.70 (0.66-0.73) | 0.66 (0.61-0.71) | 0.56 (0.36-0.76) | 0.26 |

[a]Geometric least squares mean (95% confidence interval). [b]Homozygous major allele *vs* heterozygous only (homozygous minor allele group not included, because it contains less then 10 individuals). $p_G$ = *p*-value general association model.

| | Complete sample *GAD2* (-243A>G) | | | |
|---|---|---|---|---|
| Trait | AA | AG | GG | $p_G$ |
| Birth weight | 2557 (2513-2601) | 2541 (2477-2606) | 2569 (2374-2764) | 0.90 |
| Body mass | 65.0 (64.0-66.0) | 63.7 (62.1-65.2) | 66.1 (61.7-70.5) | 0.28 |
| BMI[a] | 21.8 (21.5-22.2) | 21.5 (21.0-22.0) | 22.2 (20.7-23.7) | 0.40 |
| Fat mass[a] | 14.0 (13.5-14.6) | 13.9 (13.1-14.7) | 13.2 (11.1-15.8) | 0.78 |
| Lean body mass | 50.0 (49.5-50.6) | 48.9 (48.1-49.8) | 51.0 (48.6-53.4) | 0.05 |
| S4SF[a] | 43.6 (41.7-45.5) | 43.3 (40.5-46.2) | 37.9 (31.1-46.2) | 0.40 |
| WHR | 77.8 (77.2-78.3) | 77.2 (76.4-78.0) | 76.4 (74.1-78.7) | 0.33 |
| IGFBP1[a] | 14.2 (13.2-15.2) | 14.7 (13.1-16.3) | 13.6 (10.00-18.4) | 0.82 |
| Fasting glucose | 4.74 (4.69-4.79) | 4.70 (4.63-4.77) | 4.71 (4.51-4.92) | 0.63 |
| Fasting insulin[a] | 36.4 (34.8-38.1) | 33.1 (30.9-35.5) | 43.7 (35.7-53.6) | 0.01 |
| Leptin[a] | 4.38 (4.10-4.68) | 4.30 (3.89-4.74) | 4.41 (3.27-5.96) | 0.94 |
| Total cholesterol | 5.06 (4.95-5.17) | 4.98 (4.82-5.15) | 4.88 (4.42-5.35) | 0.63 |
| LDL-cholesterol | 2.99 (2.89-3.09) | 2.92 (2.77-3.06) | 2.92 (2.49-3.35) | 0.68 |
| HDL-cholesterol | 1.62 (1.57-1.66) | 1.63 (1.56-1.69) | 1.39 (1.21-1.58) | 0.06 |
| Triglycerides[a] | 0.88 (0.84-0.92) | 0.84 (0.78-0.90) | 0.98 (0.80-1.21) | 0.25 |
| NEFA[a] | 0.55 (0.53-0.57) | 0.56 (0.52-0.59) | 0.67 (0.56-0.81) | 0.09 |

[a]Geometric least squares mean (95% confidence interval). $p_G$ = *p*-value general association model.

| | Men only sample *GAD2* (-243A>G) | | | |
|---|---|---|---|---|
| Trait | AA | AG | GG | $p_G$[b] |
| Birth weight | 2629 (2564-2695) | 2565 (2479-2650) | 2566 (2281-2850) | 0.22 |
| Body mass | 70.2 (68.8-71.6) | 67.7 (65.8-69.5) | 68.8 (62.6-75.1) | 0.03 |
| BMI[a] | 22.1 (21.6-22.5) | 21.2 (20.6-21.8) | 21.7 (19.7-23.8) | 0.02 |
| Fat mass[a] | 12.0 (11.2-12.8) | 11.9 (10.9-13.0) | 9.67 (7.03-13.3) | 0.92 |
| Lean body mass | 57.6 (56.7-58.5) | 55.4 (54.2-56.6) | 58.5 (54.5-62.5) | 0.004 |
| S4SF[a] | 35.2 (32.8-37.8) | 33.6 (30.6-36.9) | 31.8 (22.9-44.3) | 0.42 |
| WHR | 82.8 (82.0-83.7) | 82.4 (81.3-83.6) | 81.6 (77.7-85.5) | 0.60 |
| IGFBP1[a] | 11.6 (10.6-12.6) | 12.1 (10.8-13.5) | 12.6 (8.47-18.7) | 0.55 |
| Fasting glucose | 4.90 (4.83-4.98) | 4.81 (4.71-4.91) | 4.90 (4.54-5.25) | 0.13 |
| Fasting insulin[a] | 34.0 (31.8-36.4) | 28.8 (26.3-31.6) | 36.1 (26.6-48.9) | 0.004 |
| Leptin[a] | 1.59 (1.42-1.77) | 1.51 (1.29-1.75) | 1.77 (1.05-2.99) | 0.57 |
| Total cholesterol | 4.97 (4.80-5.13) | 4.76 (4.54-4.98) | 4.05 (3.31-4.79) | 0.13 |
| LDL-cholesterol | 3.11 (2.95-3.26) | 2.99 (2.78-3.20) | 2.50 (1.79-3.21) | 0.38 |
| HDL-cholesterol[a] | 1.38 (1.32-1.44) | 1.34 (1.27-1.42) | 1.10 (0.91-1.33) | 0.42 |
| Triglycerides[a] | 0.86 (0.81-0.92) | 0.79 (0.72-0.87) | 0.70 (0.53-0.94) | 0.14 |
| NEFA[a] | 0.48 (0.45-0.51) | 0.47 (0.43-0.52) | 0.56 (0.41-0.76) | 0.87 |

[a]Geometric least squares mean (95% confidence interval). [b]Homozygous major allele *vs* heterozygous only (homozygous minor allele group not included, because it contains less then 10 individuals). $p_G$ = *p*-value general association model.

| Trait | Women only sample *GAD2* (-243A>G) AA | AG | GG | $p_G$ |
|---|---|---|---|---|
| Birth weight | 2496 (2438-2555) | 2493 (2401-2584) | 2513 (2239-2787) | 0.99 |
| Body mass | 60.1 (58.7-61.4) | 60.2 (58.0-62.4) | 64.1 (58.0-70.1) | 0.44 |
| BMI[a] | 21.6 (21.2-22.1) | 21.7 (21.0-22.5) | 23.0 (20.9-25.3) | 0.48 |
| Fat mass[a] | 16.4 (15.8-17.1) | 16.5 (15.4-17.6) | 18.2 (15.1-22.0) | 0.57 |
| Lean body mass | 43.0 (42.3-43.7) | 43.1 (41.9-44.2) | 44.7 (41.7-47.7) | 0.54 |
| S4SF[a] | 53.8 (51.0-56.7) | 55.3 (50.8-60.3) | 48.9 (38.2-62.6) | 0.61 |
| WHR | 72.7 (72.0-73.4) | 72.4 (71.3-73.4) | 71.8 (68.9-74.7) | 0.75 |
| IGFBP1[a] | 17.4 (15.7-19.4) | 17.5 (14.5-21.0) | 13.6 (8.76-21.1) | 0.55 |
| Fasting glucose | 4.57 (4.52-4.63) | 4.53 (4.44-4.63) | 4.65 (4.40-4.90) | 0.61 |
| Fasting insulin[a] | 39.3 (37.1-41.7) | 38.8 (35.2-42.8) | 52.1 (39.9-68.1) | 0.12 |
| Leptin[a] | 11.8 (11.0-12.6) | 12.4 (11.1-14.0) | 11.1 (8.03-15.5) | 0.66 |
| Total cholesterol[a] | 5.04 (4.91-5.17) | 5.09 (4.87-5.31) | 5.51 (4.91-6.19) | 0.33 |
| LDL-cholesterol | 2.89 (2.77-3.01) | 2.82 (2.63-3.02) | 3.27 (2.73-3.80) | 0.30 |
| HDL-cholesterol[a] | 1.77 (1.71-1.83) | 1.83 (1.73-1.94) | 1.62 (1.41-1.87) | 0.26 |
| Triglycerides[a] | 0.89 (0.83-0.94) | 0.90 (0.81-1.00) | 1.24 (0.93-1.65) | 0.08 |
| NEFA | 0.67 (0.63-0.70) | 0.70 (0.64-0.75) | 0.81 (0.66-0.96) | 0.14 |

[a]Geometric least squares mean (95% confidence interval). $p_G$ = $p$-value general association model.

| Trait | Complete sample *IL6* (-174G>C) GG | GC | CC | $p_G$ |
|---|---|---|---|---|
| Birth weight | 2555 (2495-2615) | 2533 (2481-2584) | 2580 (2500-2661) | 0.57 |
| Body mass | 63.9 (62.5-65.3) | 65.3 (64.1-66.5) | 64.4 (62.6-66.3) | 0.29 |
| BMI[a] | 21.5 (21.0-21.9) | 22.0 (21.6-22.4) | 21.7 (21.1-22.3) | 0.24 |
| Fat mass[a] | 13.5 (12.8-14.3) | 14.1 (13.4-14.8) | 14.4 (13.4-15.5) | 0.35 |
| Lean body mass | 49.5 (48.7-50.3) | 50.1 (49.4-50.8) | 49.3 (48.3-50.4) | 0.32 |
| S4SF[a] | 40.9 (38.6-43.4) | 44.9 (42.6-47.2) | 43.9 (40.6-47.5) | 0.06 |
| WHR | 77.5 (76.8-78.2) | 77.8 (77.2-78.4) | 77.2 (76.3-78.2) | 0.55 |
| IGFBP1[a] | 13.6 (12.3-15.0) | 15.2 (13.9-16.6) | 14.8 (12.9-17.0) | 0.25 |
| Fasting glucose | 4.74 (4.68-4.81) | 4.68 (4.63-4.73) | 4.78 (4.70-4.86) | 0.07 |
| Fasting insulin[a] | 37.0 (34.7-39.4) | 34.7 (32.9-36.7) | 34.6 (31.7-37.7) | 0.28 |
| Leptin[a] | 4.40 (4.01-4.83) | 4.15 (3.84-4.50) | 4.49 (3.98-5.07) | 0.46 |
| Total cholesterol | 4.98 (4.84-5.13) | 5.00 (4.87-5.13) | 5.04 (4.85-5.24) | 0.88 |
| LDL-cholesterol | 2.95 (2.81-3.08) | 2.95 (2.84-3.07) | 2.91 (2.73-3.08) | 0.88 |
| HDL-cholesterol | 1.60 (1.54-1.66) | 1.59 (1.54-1.64) | 1.71 (1.63-1.79) | 0.03 |
| Triglycerides[a] | 0.86 (0.81-0.92) | 0.89 (0.84-0.94) | 0.80 (0.73-0.87) | 0.11 |
| NEFA[a] | 0.56 (0.53-0.59) | 0.54 (0.52-0.57) | 0.58 (0.54-0.63) | 0.33 |

[*]Geometric least squares mean (95% confidence interval). $p_G$ = $p$-value general association model.

| Trait | Men only sample *IL6* (-174G>C) GG | GC | CC | $p_G$ |
|---|---|---|---|---|
| Birth weight | 2624 (2542-2706) | 2583 (2506-2659) | 2638 (2521-2755) | 0.64 |
| Body mass | 70.3 (68.6-72.1) | 68.8 (67.2-70.4) | 67.3 (64.8-69.9) | 0.15 |
| BMI[a] | 22.1 (21.5-22.7) | 21.5 (21.0-22.1) | 21.2 (20.5-22.0) | 0.19 |
| Fat mass[a] | 11.9 (10.9-13.0) | 11.4 (10.6-12.4) | 12.3 (11.0-13.9) | 0.51 |
| Lean body mass | 57.7 (56.5-58.8) | 56.7 (55.6-57.7) | 55.4 (53.8-57.1) | 0.09 |
| S4SF[a] | 34.9 (31.9-38.2) | 33.9 (31.2-36.8) | 34.4 (30.3-39.1) | 0.88 |
| WHR | 82.8 (81.7-83.9) | 82.6 (81.6-83.6) | 82.2 (80.7-83.7) | 0.80 |
| IGFBP1[a] | 11.6 (10.4-13.0) | 12.5 (11.3-13.9) | 11.4 (9.66-13.5) | 0.52 |
| Fasting glucose | 4.89 (4.79-4.99) | 4.82 (4.73-4.90) | 4.91 (4.77-5.04) | 0.38 |
| Fasting insulin[a] | 34.4 (31.5-37.5) | 31.2 (28.8-33.9) | 29.7 (26.1-33.8) | 0.13 |
| Leptin[a] | 1.69 (1.46-1.95) | 1.44 (1.26-1.64) | 1.45 (1.18-1.79) | 0.24 |
| Total cholesterol | 4.86 (4.65-5.07) | 4.77 (4.58-4.96) | 4.97 (4.67-5.27) | 0.51 |
| LDL-cholesterol | 3.06 (2.86-3.26) | 3.00 (2.81-3.18) | 2.99 (2.71-3.27) | 0.86 |
| HDL-cholesterol[a] | 1.32 (1.25-1.40) | 1.35 (1.28-1.42) | 1.44 (1.33-1.56) | 0.20 |
| Triglycerides[a] | 0.84 (0.77-0.92) | 0.83 (0.76-0.90) | 0.79 (0.70-0.89) | 0.68 |
| NEFA[a] | 0.46 (0.43-0.50) | 0.47 (0.43-0.50) | 0.56 (0.50-0.64) | 0.02 |

[a]Geometric least squares mean (95% confidence interval). $p_G$ = *p*-value general association model.

| Trait | Women only sample *IL6* (-174G>C) GG | GC | CC | $p_G$ |
|---|---|---|---|---|
| Birth weight | 2479 (2392-2565) | 2475 (2406-2544) | 2516 (2407-2626) | 0.80 |
| Body mass | 58.1 (56.1-60.1) | 61.6 (60.0-63.2) | 60.7 (58.2-63.1) | 0.03 |
| BMI[a] | 21.0 (20.3-21.7) | 22.3 (21.7-22.9) | 21.7 (20.9-22.6) | 0.02 |
| Fat mass[a] | 15.4 (14.6-16.4) | 17.0 (16.2-17.9) | 17.2 (16.0-18.5) | 0.02 |
| Lean body mass | 42.1 (41.1-43.1) | 43.7 (42.8-44.5) | 43.1 (41.8-44.4) | 0.06 |
| S4SF[a] | 49.1 (45.6-53.0) | 57.8 (54.4-61.4) | 54.7 (49.7-60.1) | 0.005 |
| WHR | 72.5 (71.5-73.4) | 72.9 (72.1-73.7) | 72.2 (70.9-73.4) | 0.58 |
| IGFBP1[a] | 16.0 (13.6-18.8) | 18.3 (16.1-20.9) | 18.8 (15.2-23.2) | 0.35 |
| Fasting glucose | 4.54 (4.46-4.62) | 4.53 (4.46-4.60) | 4.66 (4.56-4.77) | 0.08 |
| Fasting insulin[a] | 40.3 (36.8-44.2) | 39.1 (36.4-42.1) | 39.4 (35.3-44.0) | 0.87 |
| Leptin[a] | 11.1 (9.94-12.4) | 12.0 (11.0-13.1) | 13.3 (11.7-15.2) | 0.11 |
| Total cholesterol[a] | 5.03 (4.84-5.23) | 5.09 (4.93-5.25) | 5.01 (4.77-5.26) | 0.81 |
| LDL-cholesterol | 2.83 (2.65-3.00) | 2.91 (2.77-3.05) | 2.78 (2.57-3.00) | 0.55 |
| HDL-cholesterol[a] | 1.78 (1.69-1.87) | 1.76 (1.69-1.84) | 1.87 (1.76-2.00) | 0.29 |
| Triglycerides[a] | 0.87 (0.79-0.95) | 0.95 (0.88-1.02) | 0.79 (0.70-0.90) | 0.05 |
| NEFA | 0.70 (0.65-0.75) | 0.69 (0.64-0.73) | 0.65 (0.58-0.71) | 0.45 |

[a]Geometric least squares mean (95% confidence interval). $p_G$ = *p*-value general association model.

| Trait | Complete sample *IRS2* (G1057D) GG | GD | DD | $p_G$ |
|---|---|---|---|---|
| Birth weight | 2538 (2483-2593) | 2538 (2486-2590) | 2675 (2568-2781) | 0.06 |
| Body mass | 64.1 (62.9-65.4) | 64.4 (63.2-65.5) | 65.0 (62.4-67.5) | 0.84 |
| BMI[a] | 21.6 (21.2-22.0) | 21.8 (21.4-22.1) | 21.8 (21.0-22.7) | 0.74 |
| Fat mass[a] | 13.7 (13.1-14.4) | 14.1 (13.5-14.7) | 13.6 (12.3-15.0) | 0.67 |
| Lean body mass | 49.5 (48.8-50.2) | 49.5 (48.8-50.2) | 50.5 (49.1-51.9) | 0.42 |
| S4SF[a] | 43.0 (40.7-45.3) | 43.0 (41.0-45.2) | 43.0 (38.6-48.0) | 1.00 |
| WHR | 77.2 (76.6-77.9) | 77.8 (77.2-78.4) | 77.6 (76.3-78.9) | 0.42 |
| IGFBP1[a] | 15.2 (13.8-16.6) | 13.8 (12.7-15.0) | 14.5 (12.1-17.3) | 0.31 |
| Fasting glucose | 4.78 (4.72-4.84) | 4.70 (4.64-4.75) | 4.65 (4.53-4.77) | 0.05 |
| Fasting insulin[a] | 35.4 (33.4-37.5) | 36.2 (34.4-38.2) | 35.4 (31.5-39.7) | 0.82 |
| Leptin[a] | 4.60 (4.23-5.00) | 4.32 (4.01-4.66) | 4.02 (3.41-4.75) | 0.30 |
| Total cholesterol | 5.02 (4.88-5.15) | 5.00 (4.88-5.13) | 5.08 (4.81-5.34) | 0.88 |
| LDL-cholesterol | 2.93 (2.81-3.06) | 2.96 (2.85-3.07) | 3.03 (2.79-3.27) | 0.79 |
| HDL-cholesterol | 1.64 (1.58-1.70) | 1.61 (1.56-1.66) | 1.61 (1.50-1.72) | 0.70 |
| Triglycerides[a] | 0.87 (0.82-0.92) | 0.86 (0.82-0.91) | 0.88 (0.78-0.99) | 0.94 |
| NEFA[a] | 0.54 (0.51-0.57) | 0.58 (0.55-0.61) | 0.53 (0.48-0.59) | 0.14 |

[a]Geometric least squares mean (95% confidence interval). $p_G$ = *p*-value general association model.

| Trait | Men only sample *IRS2* (G1057D) GG | GD | DD | $p_G$ |
|---|---|---|---|---|
| Birth weight | 2594 (2516-2673) | 2576 (2501-2651) | 2765 (2616-2914) | 0.08 |
| Body mass | 69.3 (67.6-71.0) | 68.8 (67.2-70.3) | 71.2 (68.0-74.3) | 0.38 |
| BMI[a] | 21.9 (21.4-22.4) | 21.5 (21.0-22.0) | 22.2 (21.2-23.3) | 0.32 |
| Fat mass[a] | 11.7 (10.8-12.6) | 11.7 (10.9-12.6) | 12.6 (10.8-14.6) | 0.67 |
| Lean body mass | 57.0 (55.9-58.1) | 56.4 (55.3-57.4) | 58.2 (56.1-60.3) | 0.27 |
| S4SF[a] | 34.8 (31.9-37.8) | 33.4 (30.9-36.1) | 37.0 (31.7-43.2) | 0.44 |
| WHR | 82.1 (81.1-83.2) | 83.1 (82.1-84.0) | 82.7 (80.8-84.6) | 0.38 |
| IGFBP1[a] | 12.1 (10.8-13.4) | 11.7 (10.6-12.9) | 12.2 (9.96-14.9) | 0.88 |
| Fasting glucose | 4.93 (4.84-5.03) | 4.83 (4.74-4.91) | 4.74 (4.57-4.92) | 0.10 |
| Fasting insulin[a] | 34.4 (31.6-37.4) | 31.2 (28.9-33.7) | 30.6 (26.2-35.8) | 0.19 |
| Leptin[a] | 1.81 (1.58-2.08) | 1.44 (1.27-1.63) | 1.40 (1.08-1.80) | 0.03 |
| Total cholesterol | 4.94 (4.74-5.14) | 4.81 (4.62-5.00) | 4.85 (4.48-5.22) | 0.62 |
| LDL-cholesterol | 3.10 (2.90-3.29) | 3.00 (2.82-3.18) | 3.05 (2.69-3.41) | 0.77 |
| HDL-cholesterol[a] | 1.38 (1.31-1.46) | 1.33 (1.27-1.40) | 1.40 (1.27-1.54) | 0.48 |
| Triglycerides[a] | 0.84 (0.77-0.91) | 0.84 (0.78-0.91) | 0.82 (0.71-0.96) | 0.96 |
| NEFA[a] | 0.47 (0.43-0.51) | 0.49 (0.46-0.53) | 0.46 (0.40-0.54) | 0.65 |

[a]Geometric least squares mean (95% confidence interval). $p_G$ = *p*-value general association model.

| | Women only sample *IRS2* (G1057D) | | | |
|---|---|---|---|---|
| Trait | GG | GD | DD | $p_G$ |
| Birth weight | 2480 (2405-2556) | 2494 (2426-2563) | 2550 (2397-2704) | 0.72 |
| Body mass | 59.4 (57.7-61.1) | 60.5 (58.9-62.1) | 59.0 (55.2-62.8) | 0.58 |
| BMI[a] | 21.4 (20.8-22.0) | 21.8 (21.3-22.4) | 21.3 (20.0-22.6) | 0.51 |
| Fat mass[a] | 16.3 (15.5-17.1) | 16.9 (16.1-17.7) | 15.2 (13.6-17.0) | 0.19 |
| Lean body mass | 42.7 (41.8-43.6) | 42.9 (42.1-43.7) | 43.2 (41.2-45.1) | 0.86 |
| S4SF[a] | 53.8 (50.3-57.5) | 54.7 (51.5-58.1) | 48.7 (42.1-56.4) | 0.36 |
| WHR | 72.5 (71.6-73.3) | 72.6 (71.9-73.4) | 72.5 (70.7-74.3) | 0.96 |
| IGFBP1[a] | 19.1 (16.6-22.1) | 16.2 (14.3-18.4) | 17.3 (13.1-22.9) | 0.24 |
| Fasting glucose | 4.61 (4.54-4.69) | 4.57 (4.50-4.63) | 4.46 (4.31-4.61) | 0.20 |
| Fasting insulin[a] | 36.7 (34.1-39.6) | 41.3 (38.6-44.2) | 40.8 (34.6-48.1) | 0.07 |
| Leptin[a] | 11.8 (10.7-13.0) | 12.2 (11.2-13.3) | 9.95 (8.15-12.2) | 0.19 |
| Total cholesterol[a] | 5.00 (4.82-5.17) | 5.09 (4.94-5.25) | 5.10 (4.74-5.48) | 0.70 |
| LDL-cholesterol | 2.78 (2.63-2.93) | 2.91 (2.77-3.04) | 2.91 (2.59-3.23) | 0.44 |
| HDL-cholesterol[a] | 1.79 (1.71-1.88) | 1.80 (1.73-1.88) | 1.77 (1.61-1.95) | 0.94 |
| Triglycerides[a] | 0.90 (0.82-0.98) | 0.87 (0.81-0.94) | 0.90 (0.75-1.07) | 0.86 |
| NEFA | 0.66 (0.61-0.70) | 0.71 (0.67-0.75) | 0.64 (0.55-0.74) | 0.14 |

[a]Geometric least squares mean (95% confidence interval). $p_G$ = *p*-value general association model.

| | Complete sample *KCNJ11* (E23K) | | | |
|---|---|---|---|---|
| Trait | EE | EK | KK | $p_G$ |
| Birth weight | 2588 (2531-2644) | 2528 (2476-2579) | 2573 (2477-2669) | 0.25 |
| Body mass | 64.2 (62.9-65.5) | 65.0 (63.8-66.2) | 63.7 (61.5-66.0) | 0.49 |
| BMI[a] | 21.7 (21.2-22.1) | 21.9 (21.5-22.3) | 21.5 (20.8-22.2) | 0.49 |
| Fat mass[a] | 13.9 (13.3-14.7) | 14.2 (13.5-14.9) | 13.2 (12.1-14.4) | 0.37 |
| Lean body mass | 49.4 (48.6-50.1) | 50.0 (49.3-50.6) | 49.3 (48.1-50.5) | 0.40 |
| S4SF[a] | 42.8 (40.4-45.2) | 44.3 (42.1-46.7) | 41.4 (37.6-45.5) | 0.36 |
| WHR | 77.4 (76.7-78.1) | 77.9 (77.2-78.5) | 77.4 (76.2-78.5) | 0.55 |
| IGFBP1[a] | 14.3 (13.0-15.7) | 14.7 (13.5-16.0) | 13.6 (11.6-16.0) | 0.69 |
| Fasting glucose | 4.72 (4.66-4.78) | 4.74 (4.68-4.79) | 4.61 (4.51-4.71) | 0.10 |
| Fasting insulin[a] | 35.1 (33.1-37.3) | 36.3 (34.3-38.3) | 33.7 (30.3-37.4) | 0.42 |
| Leptin[a] | 4.57 (4.20-4.97) | 4.46 (4.12-4.82) | 3.75 (3.24-4.33) | 0.06 |
| Total cholesterol | 5.05 (4.92-5.19) | 5.02 (4.89-5.15) | 4.95 (4.71-5.18) | 0.72 |
| LDL-cholesterol | 2.96 (2.83-3.08) | 2.97 (2.86-3.09) | 3.04 (2.82-3.25) | 0.81 |
| HDL-cholesterol | 1.65 (1.59-1.71) | 1.61 (1.55-1.66) | 1.53 (1.44-1.63) | 0.10 |
| Triglycerides[a] | 0.86 (0.81-0.92) | 0.88 (0.83-0.93) | 0.84 (0.75-0.93) | 0.75 |
| NEFA[a] | 0.55 (0.52-0.58) | 0.55 (0.52-0.58) | 0.59 (0.54-0.65) | 0.34 |

[a]Geometric least squares mean (95% confidence interval). $p_G$ = *p*-value general association model.

| | Men only sample *KCNJ11* (E23K) | | | |
|---|---|---|---|---|
| Trait | EE | EK | KK | $p_G$ |
| Birth weight | 2655 (2570-2740) | 2597 (2520-2673) | 2580 (2451-2709) | 0.49 |
| Body mass | 69.2 (67.5-70.9) | 69.8 (68.3-71.4) | 67.1 (64.4-69.7) | 0.20 |
| BMI[a] | 21.7 (21.2-22.3) | 22.0 (21.5-22.5) | 21.1 (20.3-21.9) | 0.16 |
| Fat mass[a] | 11.7 (10.8-12.7) | 12.1 (11.3-13.1) | 11.2 (9.88-12.8) | 0.56 |
| Lean body mass | 56.5 (55.4-57.7) | 57.3 (56.2-58.3) | 56.1 (54.3-57.9) | 0.44 |
| S4SF[a] | 34.3 (31.4-37.4) | 35.4 (32.7-38.3) | 31.3 (27.4-35.8) | 0.28 |
| WHR | 82.5 (81.5-83.6) | 83.2 (82.2-84.1) | 81.1 (79.5-82.7) | 0.08 |
| IGFBP1[a] | 11.8 (10.6-13.2) | 12.4 (11.2-13.7) | 11.8 (9.88-14.1) | 0.81 |
| Fasting glucose | 4.87 (4.77-4.97) | 4.91 (4.82-5.00) | 4.71 (4.56-4.86) | 0.07 |
| Fasting insulin[a] | 30.6 (27.9-33.5) | 33.8 (31.1-36.7) | 30.6 (26.5-35.2) | 0.21 |
| Leptin[a] | 1.65 (1.43-1.91) | 1.62 (1.42-1.85) | 1.18 (0.95-1.48) | 0.04 |
| Total cholesterol | 4.79 (4.59-5.00) | 4.91 (4.73-5.10) | 4.78 (4.47-5.10) | 0.61 |
| LDL-cholesterol | 2.91 (2.71-3.11) | 3.11 (2.93-3.29) | 3.09 (2.78-3.40) | 0.27 |
| HDL-cholesterol[a] | 1.41 (1.34-1.49) | 1.34 (1.28-1.41) | 1.28 (1.17-1.39) | 0.11 |
| Triglycerides[a] | 0.82 (0.75-0.90) | 0.83 (0.77-0.89) | 0.87 (0.76-0.99) | 0.77 |
| NEFA[a] | 0.47 (0.43-0.51) | 0.47 (0.44-0.51) | 0.55 (0.48-0.63) | 0.12 |

[a]Geometric least squares mean (95% confidence interval). $p_G$ = *p*-value general association model.

| | Women only sample *KCNJ11* (E23K) | | | |
|---|---|---|---|---|
| Trait | EE | EK | KK | $p_G$ |
| Birth weight | 2500 (2427-2573) | 2473 (2405-2541) | 2554 (2416-2693) | 0.55 |
| Body mass | 60.0 (58.2-61.8) | 60.4 (58.8-62.1) | 61.5 (58.2-64.9) | 0.72 |
| BMI[a] | 21.7 (21.1-22.3) | 21.8 (21.2-22.4) | 22.0 (20.9-23.2) | 0.91 |
| Fat mass[a] | 16.5 (15.6-17.4) | 16.6 (15.8-17.5) | 16.9 (15.3-18.6) | 0.92 |
| Lean body mass | 42.8 (41.9-43.7) | 43.3 (42.4-44.1) | 43.4 (41.8-45.1) | 0.67 |
| S4SF[a] | 53.5 (49.9-57.4) | 55.3 (51.8-59.0) | 54.2 (47.6-61.8) | 0.79 |
| WHR | 72.4 (71.5-73.2) | 72.7 (71.9-73.5) | 73.6 (71.9-75.2) | 0.43 |
| IGFBP1[a] | 17.1 (14.9-19.7) | 17.4 (15.3-19.8) | 15.8 (12.1-20.5) | 0.80 |
| Fasting glucose | 4.57 (4.50-4.64) | 4.56 (4.49-4.63) | 4.45 (4.32-4.59) | 0.29 |
| Fasting insulin[a] | 39.6 (36.6-42.8) | 39.6 (36.8-42.6) | 38.2 (32.9-44.2) | 0.90 |
| Leptin[a] | 12.2 (11.2-13.4) | 11.9 (10.9-13.0) | 12.1 (10.2-14.4) | 0.90 |
| Total cholesterol[a] | 5.13 (4.95-5.31) | 5.04 (4.88-5.20) | 5.13 (4.81-5.47) | 0.72 |
| LDL-cholesterol | 2.94 (2.79-3.10) | 2.82 (2.68-2.96) | 3.09 (2.80-3.37) | 0.18 |
| HDL-cholesterol[a] | 1.81 (1.73-1.89) | 1.77 (1.70-1.85) | 1.73 (1.59-1.88) | 0.63 |
| Triglycerides[a] | 0.88 (0.81-0.96) | 0.92 (0.85-0.99) | 0.81 (0.69-0.95) | 0.35 |
| NEFA | 0.68 (0.64-0.72) | 0.68 (0.64-0.72) | 0.66 (0.57-0.74) | 0.85 |

[a]Geometric least squares mean (95% confidence interval). $p_G$ = *p*-value general association model.

| Trait | Complete sample *PPARγ* (P12A) | | | |
|---|---|---|---|---|
| | PP | PA | AA | $p_G$[b] |
| Birth weight | 2528 (2487-2569) | 2614 (2537-2691) | 2339 (1984-2695) | 0.04 |
| Body mass | 64.2 (63.3-65.2) | 65.3 (63.5-67.1) | 68.8 (60.3-77.2) | 0.25 |
| BMI[a] | 21.6 (21.3-21.9) | 22.0 (21.4-22.6) | 23.4 (20.6-26.5) | 0.21 |
| Fat mass[a] | 13.6 (13.1-14.1) | 14.8 (13.8-15.9) | 18.3 (13.1-25.6) | 0.03 |
| Lean body mass | 49.7 (49.2-50.2) | 49.4 (48.4-50.5) | 49.2 (44.5-53.9) | 0.77 |
| S4SF[a] | 42.4 (40.7-44.1) | 45.2 (41.9-48.7) | 68.7 (48.4-97.3) | 0.11 |
| WHR | 77.3 (76.8-77.8) | 78.4 (77.5-79.3) | 80.3 (75.9-84.7) | 0.03 |
| IGFBP1[a] | 14.3 (13.3-15.2) | 14.7 (12.9-16.8) | 15.7 (7.91-31.4) | 0.65 |
| Fasting glucose | 4.73 (4.69-4.77) | 4.69 (4.61-4.77) | 5.14 (4.69-5.60) | 0.28 |
| Fasting insulin[a] | 36.2 (34.7-37.7) | 34.1 (31.4-37.0) | 33.8 (22.3-51.4) | 0.18 |
| Leptin[a] | 4.33 (4.08-4.60) | 4.23 (3.77-4.75) | 6.43 (3.66-11.3) | 0.67 |
| Total cholesterol | 5.03 (4.93-5.14) | 4.97 (4.78-5.16) | 4.57 (3.65-5.50) | 0.48 |
| LDL-cholesterol | 2.95 (2.86-3.04) | 2.96 (2.79-3.13) | 2.52 (1.72-3.32) | 0.99 |
| HDL-cholesterol | 1.63 (1.59-1.67) | 1.59 (1.51-1.67) | 1.59 (1.21-1.98) | 0.44 |
| Triglycerides[a] | 0.86 (0.83-0.90) | 0.87 (0.80-0.94) | 0.67 (0.43-1.05) | 0.98 |
| NEFA[a] | 0.56 (0.54-0.58) | 0.54 (0.50-0.58) | 0.50 (0.33-0.76) | 0.41 |

[a]Geometric least squares mean (95% confidence interval). [b]Homozygous major allele *vs* heterozygous only (homozygous minor allele group not included, because it contains less then 10 individuals). $p_G$ = *p*-value general association model.

| Trait | Men only sample *PPARγ* (P12A) | | | |
|---|---|---|---|---|
| | PP | PA | AA | $p_G$[b] |
| Birth weight | 2579 (2521-2637) | 2663 (2548-2777) | 2353 (1906-2801) | 0.19 |
| Body mass | 68.8 (67.6-70.0) | 69.9 (67.5-72.2) | 67.3 (57.1-77.5) | 0.42 |
| BMI[a] | 21.6 (21.2-22.0) | 21.9 (21.1-22.7) | 21.2 (18.2-24.7) | 0.58 |
| Fat mass[a] | 11.4 (10.8-12.1) | 12.9 (11.5-14.4) | 12.0 (7.44-19.2) | 0.05 |
| Lean body mass | 57.0 (56.2-57.8) | 56.1 (54.5-57.7) | 52.3 (45.5-59.1) | 0.31 |
| S4SF[a] | 33.5 (31.5-35.7) | 36.7 (32.6-41.3) | 44.8 (26.8-74.7) | 0.16 |
| WHR | 82.4 (81.6-83.1) | 83.3 (81.9-84.7) | 82.9 (76.9-89.0) | 0.22 |
| IGFBP1[a] | 11.9 (11.0-12.8) | 12.7 (11.0-14.8) | 11.0 (5.84-20.6) | 0.42 |
| Fasting glucose | 4.88 (4.82-4.95) | 4.76 (4.64-4.88) | 4.97 (4.43-5.50) | 0.07 |
| Fasting insulin[a] | 33.1 (31.1-35.1) | 29.3 (26.0-33.1) | 40.4 (24.3-67.4) | 0.08 |
| Leptin[a] | 1.60 (1.45-1.77) | 1.30 (1.07-1.58) | 2.84 (1.24-6.50) | 0.05 |
| Total cholesterol | 4.88 (4.73-5.03) | 4.71 (4.43-4.99) | 5.08 (3.84-6.32) | 0.29 |
| LDL-cholesterol | 3.03 (2.89-3.17) | 3.00 (2.73-3.27) | 2.99 (1.80-4.18) | 0.83 |
| HDL-cholesterol[a] | 1.37 (1.32-1.43) | 1.30 (1.20-1.40) | 1.67 (1.20-2.33) | 0.15 |
| Triglycerides[a] | 0.82 (0.77-0.87) | 0.85 (0.76-0.96) | 0.71 (0.41-1.23) | 0.52 |
| NEFA[a] | 0.48 (0.45-0.51) | 0.49 (0.43-0.55) | 0.46 (0.27-0.79) | 0.80 |

[a]Geometric least squares mean (95% confidence interval). [b]Homozygous major allele *vs* heterozygous only (homozygous minor allele group not included, because it contains less then 10 individuals). $p_G$ = *p*-value general association model.

| Trait | Women only sample *PPARγ* (P12A) | | | $p_G$[b] |
|---|---|---|---|---|
| | PP | PA | AA | |
| Birth weight | 2472 (2418-2526) | 2568 (2463-2674) | 2388 (1660-3116) | 0.11 |
| Body mass | 59.7 (58.5-61.0) | 61.8 (59.3-64.3) | 73.1 (57.7-88.5) | 0.14 |
| BMI[a] | 21.6 (21.1-22.0) | 22.2 (21.3-23.1) | 27.6 (21.5-35.3) | 0.20 |
| Fat mass[a] | 16.2 (15.6-16.9) | 17.6 (16.3-18.9) | 23.1 (14.7-36.2) | 0.06 |
| Lean body mass | 42.9 (42.2-43.5) | 43.5 (42.2-44.7) | 50.0 (42.0-57.9) | 0.41 |
| S4SF[a] | 53.2 (50.6-55.9) | 56.6 (51.5-62.2) | 110 (59.7-202) | 0.25 |
| WHR | 72.3 (71.7-72.9) | 73.6 (72.4-74.8) | 77.1 (69.1-85.2) | 0.06 |
| IGFBP1[a] | 17.3 (15.7-19.2) | 17.1 (14.1-20.8) | 36.4 (7.92-168) | 0.91 |
| Fasting glucose | 4.57 (4.52-4.63) | 4.57 (4.47-4.67) | 4.27 (3.60-4.93) | 0.92 |
| Fasting insulin[a] | 39.7 (37.5-41.9) | 39.4 (35.4-43.8) | 21.2 (10.6-42.5) | 0.90 |
| Leptin[a] | 11.5 (10.8-12.3) | 12.6 (11.1-14.3) | 11.3 (4.91-26.0) | 0.20 |
| Total cholesterol[a] | 5.08 (4.96-5.21) | 5.03 (4.80-5.27) | 3.15 (2.27-4.39) | 0.69 |
| LDL-cholesterol | 2.87 (2.76-2.98) | 2.88 (2.67-3.09) | 1.28 (-0.22-2.78) | 0.91 |
| HDL-cholesterol[a] | 1.79 (1.74-1.85) | 1.78 (1.67-1.89) | 1.66 (1.08-2.54) | 0.77 |
| Triglycerides[a] | 0.91 (0.85-0.96) | 0.87 (0.77-0.97) | 0.40 (0.17-0.94) | 0.49 |
| NEFA | 0.70 (0.66-0.73) | 0.64 (0.57-0.70) | 0.54 (0.11-0.97) | 0.10 |

[a]Geometric least squares mean (95% confidence interval). [b]Homozygous major allele *vs* heterozygous only (homozygous minor allele group not included, because it contains less then 10 individuals). $p_G$ = *p*-value general association model.

| Trait | Complete sample *PYY* (R72T) | | | $p_G$ |
|---|---|---|---|---|
| | RR | RT | TT | |
| Birth weight | 2544 (2489-2599) | 2583 (2533-2634) | 2476 (2380-2572) | 0.12 |
| Body mass | 64.5 (63.2-65.8) | 64.6 (63.4-65.7) | 64.6 (62.3-66.9) | 1.00 |
| BMI[a] | 21.7 (21.3-22.1) | 21.7 (21.3-22.1) | 21.8 (21.0-22.5) | 0.98 |
| Fat mass[a] | 13.4 (12.8-14.1) | 14.1 (13.5-14.8) | 14.3 (13.1-15.7) | 0.25 |
| Lean body mass | 49.9 (49.2-50.6) | 49.6 (49.0-50.3) | 49.2 (47.9-50.4) | 0.61 |
| S4SF[a] | 42.6 (40.3-45.0) | 43.6 (41.5-45.9) | 44.5 (40.4-49.1) | 0.68 |
| WHR | 77.3 (76.7-78.0) | 77.8 (77.2-78.4) | 77.8 (76.6-79.0) | 0.59 |
| IGFBP1[a] | 14.9 (13.5-16.3) | 13.9 (12.8-15.2) | 15.7 (13.2-18.6) | 0.36 |
| Fasting glucose | 4.74 (4.68-4.80) | 4.70 (4.65-4.76) | 4.74 (4.63-4.84) | 0.62 |
| Fasting insulin[a] | 34.8 (32.8-36.9) | 36.3 (34.5-38.3) | 35.9 (32.3-40.0) | 0.55 |
| Leptin[a] | 4.17 (3.83-4.54) | 4.45 (4.12-4.80) | 4.52 (3.88-5.25) | 0.45 |
| Total cholesterol | 5.00 (4.86-5.14) | 5.01 (4.88-5.13) | 5.07 (4.83-5.31) | 0.87 |
| LDL-cholesterol | 2.94 (2.81-3.06) | 2.98 (2.87-3.09) | 2.96 (2.75-3.17) | 0.85 |
| HDL-cholesterol | 1.63 (1.57-1.69) | 1.59 (1.54-1.64) | 1.65 (1.55-1.74) | 0.35 |
| Triglycerides[a] | 0.85 (0.80-0.90) | 0.89 (0.84-0.94) | 0.84 (0.76-0.94) | 0.42 |
| NEFA[a] | 0.56 (0.53-0.59) | 0.56 (0.53-0.59) | 0.55 (0.50-0.60) | 0.87 |

[a]Geometric least squares mean (95% confidence interval). $p_G$ = *p*-value general association model.

| | Men only sample *PYY* (R72T) | | | |
|---|---|---|---|---|
| Trait | RR | RT | TT | $p_G$ |
| Birth weight | 2618 (2540-2697) | 2622 (2545-2700) | 2508 (2371-2646) | 0.32 |
| Body mass | 69.1 (67.4-70.8) | 69.3 (67.7-71.0) | 68.7 (65.8-71.6) | 0.92 |
| BMI[a] | 21.7 (21.2-22.3) | 21.7 (21.2-22.2) | 21.5 (20.6-22.5) | 0.93 |
| Fat mass[a] | 11.4 (10.5-12.4) | 12.0 (11.1-12.9) | 11.9 (10.3-13.8) | 0.64 |
| Lean body mass | 57.3 (56.1-58.4) | 56.6 (55.5-57.7) | 56.5 (54.5-58.5) | 0.64 |
| S4SF[a] | 33.7 (31.0-36.8) | 34.7 (32.0-37.7) | 34.3 (29.5-39.9) | 0.88 |
| WHR | 82.0 (81.0-83.0) | 83.0 (82.1-84.0) | 82.5 (80.7-84.3) | 0.33 |
| IGFBP1[a] | 12.5 (11.2-14.0) | 11.5 (10.4-12.8) | 11.8 (9.78-14.2) | 0.54 |
| Fasting glucose | 4.92 (4.82-5.01) | 4.87 (4.78-4.96) | 4.70 (4.54-4.86) | 0.07 |
| Fasting insulin[a] | 31.6 (29.0-34.5) | 32.6 (30.0-35.5) | 32.5 (28.0-37.8) | 0.87 |
| Leptin[a] | 1.49 (1.29-1.71) | 1.53 (1.34-1.75) | 1.60 (1.25-2.04) | 0.87 |
| Total cholesterol | 4.92 (4.72-5.13) | 4.70 (4.51-4.89) | 5.00 (4.65-5.36) | 0.15 |
| LDL-cholesterol | 3.09 (2.89-3.28) | 2.92 (2.74-3.10) | 3.15 (2.81-3.49) | 0.31 |
| HDL-cholesterol[a] | 1.39 (1.32-1.47) | 1.31 (1.24-1.37) | 1.37 (1.25-1.50) | 0.20 |
| Triglycerides[a] | 0.81 (0.75-0.88) | 0.83 (0.76-0.90) | 0.88 (0.76-1.01) | 0.68 |
| NEFA[a] | 0.47 (0.43-0.50) | 0.49 (0.45-0.53) | 0.53 (0.46-0.61) | 0.32 |

[a]Geometric least squares mean (95% confidence interval). $p_G$ = *p*-value general association model.

| | Women only sample *PYY* (R72T) | | | |
|---|---|---|---|---|
| Trait | RR | RT | TT | $p_G$ |
| Birth weight | 2463 (2387-2539) | 2536 (2472-2601) | 2450 (2311-2590) | 0.26 |
| Body mass | 60.0 (58.1-61.8) | 60.6 (59.0-62.1) | 60.9 (57.5-64.4) | 0.83 |
| BMI[a] | 21.6 (21.0-22.3) | 21.8 (21.2-22.3) | 22.1 (21.0-23.4) | 0.77 |
| Fat mass[a] | 16.3 (15.4-17.2) | 16.7 (15.9-17.5) | 17.4 (15.7-19.2) | 0.54 |
| Lean body mass | 43.0 (42.1-43.9) | 43.1 (42.4-43.9) | 43.1 (41.4-44.8) | 0.97 |
| S4SF[a] | 53.1 (49.5-57.1) | 54.8 (51.6-58.3) | 57.8 (50.8-65.8) | 0.51 |
| WHR | 72.6 (71.7-73.5) | 72.7 (71.9-73.5) | 73.0 (71.4-74.6) | 0.91 |
| IGFBP1[a] | 17.4 (15.0-20.1) | 16.9 (14.9-19.1) | 19.9 (15.2-26.0) | 0.54 |
| Fasting glucose | 4.55 (4.47-4.62) | 4.53 (4.47-4.60) | 4.76 (4.63-4.90) | 0.01 |
| Fasting insulin[a] | 38.3 (35.4-41.5) | 40.6 (37.9-43.4) | 39.6 (34.0-46.0) | 0.56 |
| Leptin[a] | 11.2 (10.2-12.3) | 12.3 (11.4-13.4) | 12.5 (10.4-15.0) | 0.25 |
| Total cholesterol[a] | 4.95 (4.78-5.13) | 5.17 (5.02-5.32) | 5.03 (4.72-5.36) | 0.16 |
| LDL-cholesterol | 2.77 (2.62-2.93) | 2.97 (2.84-3.10) | 2.82 (2.54-3.10) | 0.15 |
| HDL-cholesterol[a] | 1.78 (1.70-1.86) | 1.77 (1.70-1.84) | 1.85 (1.70-2.01) | 0.68 |
| Triglycerides[a] | 0.88 (0.81-0.96) | 0.94 (0.87-1.01) | 0.79 (0.67-0.93) | 0.12 |
| NEFA | 0.71 (0.67-0.76) | 0.68 (0.64-0.72) | 0.61 (0.53-0.70) | 0.12 |

[*]Geometric least squares mean (95% confidence interval). $p_G$ = *p*-value general association model.

| | Complete sample *UCP2* (-866G>A) | | | |
|---|---|---|---|---|
| Trait | GG | GA | AA | $p_G$ |
| Birth weight | 2559 (2505-2613) | 2543 (2492-2595) | 2538 (2429-2648) | 0.89 |
| Body mass | 64.6 (63.4-65.9) | 64.3 (63.1-65.5) | 64.4 (61.8-66.9) | 0.94 |
| BMI[a] | 21.8 (21.4-22.3) | 21.6 (21.2-22.0) | 21.7 (20.9-22.6) | 0.69 |
| Fat mass[a] | 13.9 (13.2-14.5) | 13.8 (13.2-14.5) | 14.6 (13.2-16.1) | 0.64 |
| Lean body mass | 50.0 (49.3-50.7) | 49.6 (48.9-50.2) | 48.5 (47.1-50.0) | 0.20 |
| S4SF[a] | 43.3 (41.0-45.6) | 42.7 (40.6-44.9) | 44.9 (40.2-50.2) | 0.71 |
| WHR | 77.7 (77.1-78.4) | 77.5 (76.9-78.1) | 78.1 (76.7-79.5) | 0.68 |
| IGFBP1[a] | 14.6 (13.4-16.0) | 13.6 (12.5-14.9) | 15.5 (12.9-18.7) | 0.32 |
| Fasting glucose | 4.71 (4.66-4.77) | 4.75 (4.69-4.80) | 4.72 (4.61-4.84) | 0.67 |
| Fasting insulin[a] | 35.5 (33.5-37.6) | 35.6 (33.7-37.6) | 33.9 (30.0-38.2) | 0.76 |
| Leptin[a] | 4.33 (3.99-4.70) | 4.35 (4.02-4.70) | 4.27 (3.60-5.08) | 0.98 |
| Total cholesterol | 4.97 (4.84-5.10) | 5.06 (4.93-5.19) | 5.12 (4.84-5.39) | 0.49 |
| LDL-cholesterol | 2.91 (2.79-3.03) | 3.01 (2.90-3.12) | 3.03 (2.79-3.28) | 0.40 |
| HDL-cholesterol | 1.61 (1.55-1.66) | 1.61 (1.56-1.66) | 1.64 (1.53-1.75) | 0.89 |
| Triglycerides[a] | 0.87 (0.82-0.92) | 0.85 (0.81-0.90) | 0.88 (0.78-0.99) | 0.87 |
| NEFA[a] | 0.55 (0.52-0.58) | 0.55 (0.52-0.58) | 0.56 (0.51-0.63) | 0.93 |

[a]Geometric least squares mean (95% confidence interval). $p_G$ = *p*-value general association model.

| | Men only sample *UCP2* (-866G>A) | | | |
|---|---|---|---|---|
| Trait | GG | GA | AA | $p_G$ |
| Birth weight | 2646 (2562-2731) | 2581 (2510-2652) | 2554 (2400-2707) | 0.39 |
| Body mass | 69.2 (67.4-71.0) | 69.4 (67.9-70.9) | 68.0 (64.7-71.3) | 0.73 |
| BMI[a] | 21.9 (21.3-22.5) | 21.7 (21.2-22.2) | 21.3 (20.3-22.4) | 0.64 |
| Fat mass[a] | 11.5 (10.6-12.5) | 12.0 (11.1-12.9) | 12.2 (10.5-14.2) | 0.73 |
| Lean body mass | 57.2 (56.0-58.4) | 57.0 (55.9-58.0) | 55.1 (52.9-57.3) | 0.22 |
| S4SF[a] | 34.0 (31.0-37.2) | 34.5 (31.9-37.2) | 35.8 (30.5-42.1) | 0.85 |
| WHR | 82.4 (81.3-83.5) | 82.7 (81.8-83.6) | 83.8 (81.9-85.8) | 0.44 |
| IGFBP1[a] | 12.5 (11.2-13.9) | 10.8 (9.84-11.8) | 13.8 (11.4-16.9) | 0.03 |
| Fasting glucose | 4.87 (4.78-4.97) | 4.86 (4.78-4.94) | 4.98 (4.81-5.14) | 0.45 |
| Fasting insulin[a] | 31.8 (28.9-34.9) | 31.9 (29.6-34.5) | 35.0 (29.5-41.7) | 0.58 |
| Leptin[a] | 1.58 (1.36-1.84) | 1.53 (1.35-1.74) | 1.57 (1.19-2.08) | 0.95 |
| Total cholesterol | 4.91 (4.70-5.12) | 4.75 (4.57-4.92) | 4.95 (4.57-5.33) | 0.37 |
| LDL-cholesterol | 3.08 (2.87-3.28) | 2.94 (2.77-3.11) | 3.24 (2.88-3.60) | 0.27 |
| HDL-cholesterol[a] | 1.34 (1.27-1.42) | 1.35 (1.29-1.42) | 1.36 (1.23-1.51) | 0.96 |
| Triglycerides[a] | 0.88 (0.81-0.96) | 0.79 (0.73-0.85) | 0.81 (0.69-0.95) | 0.14 |
| NEFA[a] | 0.48 (0.44-0.52) | 0.47 (0.44-0.50) | 0.52 (0.44-0.61) | 0.55 |

[a]Geometric least squares mean (95% confidence interval). $p_G$ = *p*-value general association model.

| Trait | Women only sample *UCP2* (-866G>A) GG | GA | AA | $p_G$ |
|---|---|---|---|---|
| Birth weight | 2474 (2406-2542) | 2502 (2429-2575) | 2535 (2376-2694) | 0.72 |
| Body mass | 60.7 (59.1-62.3) | 59.1 (57.4-60.8) | 61.4 (57.6-65.3) | 0.29 |
| BMI[a] | 21.9 (21.3-22.4) | 21.4 (20.8-22.0) | 22.2 (20.8-23.6) | 0.35 |
| Fat mass[a] | 16.7 (15.9-17.5) | 16.3 (15.5-17.1) | 17.6 (15.6-19.8) | 0.46 |
| Lean body mass | 43.4 (42.6-44.2) | 42.3 (41.5-43.2) | 42.9 (41.0-44.8) | 0.18 |
| S4SF[a] | 55.5 (52.2-59.0) | 51.9 (48.6-55.4) | 55.8 (47.9-65.0) | 0.29 |
| WHR | 73.1 (72.3-73.9) | 72.2 (71.4-73.0) | 72.2 (70.4-74.1) | 0.23 |
| IGFBP1[a] | 17.3 (15.1-19.7) | 17.4 (15.1-20.0) | 17.1 (12.9-22.8) | 0.99 |
| Fasting glucose | 4.55 (4.48-4.62) | 4.59 (4.51-4.66) | 4.55 (4.40-4.71) | 0.72 |
| Fasting insulin[a] | 39.8 (37.1-42.8) | 39.6 (36.7-42.7) | 33.7 (28.4-39.9) | 0.19 |
| Leptin[a] | 11.9 (11.0-13.0) | 11.6 (10.6-12.8) | 11.6 (9.45-14.2) | 0.91 |
| Total cholesterol[a] | 4.95 (4.80-5.11) | 5.25 (5.07-5.43) | 5.12 (4.77-5.50) | 0.05 |
| LDL-cholesterol | 2.77 (2.63-2.91) | 3.07 (2.92-3.22) | 2.83 (2.51-3.14) | 0.01 |
| HDL-cholesterol[a] | 1.80 (1.72-1.87) | 1.77 (1.69-1.85) | 1.82 (1.66-2.00) | 0.78 |
| Triglycerides[a] | 0.87 (0.80-0.94) | 0.92 (0.84-1.00) | 0.95 (0.80-1.13) | 0.49 |
| NEFA | 0.67 (0.63-0.71) | 0.70 (0.66-0.74) | 0.64 (0.55-0.74) | 0.46 |

[a]Geometric least squares mean (95% confidence interval). $p_G$ = *p*-value general association model.

## 4.9 SUPPLEMENTARY MATERIAL II

In order to give an impression of the current evidence for a role in the etiology of T2D of the SNPs examined in this chapter, for each SNP a detailed overview of the studies examining the relation between the SNP and T2D and/or related traits is presented, in this supplementary file.
Since the relation between the *PPARγ* (P12A) SNP and T2D and obesity has been well established, we summarised only the results of the published meta-analysis. For the association between birth weight and *PPARγ* (P12A) we summarised all studies published thus far.
The findings of the current study are also included in the tables. In addition, the studies that are in agreement with our findings are marked with the ▲ label and the studies that are in disagreement are marked with the ▼ label. Studies that found no association got the ◆ label. BMI, S4SF, WHR, body weight and fat mass were all considered as obesity parameters, and total cholesterol, HDL-cholesterol, LDL-cholesterol and triglycerides were all considered as lipid parameters.

**Legend**
▲ Significant effect in same direction as observed in our sample.
◆ No significant effect.
▼ Significant effect in opposite direction.

**Table S5.** Summary of published studies about the association between the *PPARγ* (P12A) SNP and T2D and/or related traits.

| Author | Study design | Population | # cases | # controls | Age cases | Age controls | Definition cases | Definition controls | Allele carriers | OR | # Quant | Mean age | Geno-type | Phenotype | *p* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| ▼Deeb et al.[1a] | Quantitative | Finland | | | | | | | | | 333 | 45 | A | Lower BMI/ insulin/higher insulin sensitivity | 0.027/ 0.011/ 0.047 |
| | Quantitative | Finland | | | | | | | | | 973 | 70 | AA | Lower BMI/ insulin/higher HDL | 0.015/ 0.063/ 0.001 |
| | Case/control | Japanese-American | 155 | 54 | ? | ? | IGT | ND | PP | 2.26 | | | | | 0.073 |
| | Case/control | Japanese-American | 91 | 54 | ? | ? | T2D | ND | PP | 4.53 | | | | | 0.028 |
| ◆Altshuler et al.[2] | Parent offspring trios | Scandinavia | 333 | 379 | 37[b] | 31 | T2D & IGT | | A | 0.78 | | | | | 0.045 |
| | Case/control sib-ships | Scandinavia | 612 | 518 | 56[b] | 62 | T2D & IGT | ND | A | 0.74 | | | | | 0.071 |
| | Case/control | Scandinavia | 481 | 481 | 54[b] | 60 | T2D & IGT | ND | A | 0.88 | | | | | 0.10 |
| | Case/control | Canada | 127 | 127 | 53 | 52 | T2D | ND | A | 0.71 | | | | | 0.08 |
| | All | Scandinavia, Canada | | | | | | | P | 1.25 | | | | | 0.002 |
| | Quantitative | Scandinavia | | | | | | | | | 333 (T2D & IGT) | 39 | AA | Higher HDL BMI/glucose/ insulin parameters | 0.02 NS |
| | Quantitative | Scandinavia | | | | | | | | | 379 | 31 | | BMI/glucose/ insulin/lipid parameters | NS |
| | Meta analysis [1-7] | Japanese-American, Italy, Germany, France, Scandinavia, Canada, Japan | ? | ? | ? | ? | T2D | ND | A | 0.79 | | | | | 0.00007 |

| Author | Study design | Population | # cases | # controls | Age cases | Age controls | Definition cases | Definition controls | Allele carriers | OR | # Quant | Mean age | Geno-type | Phenotype | *p* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Lohmueller et al.[8] | Meta-analysis | 13 studies | ? | ? | ? | ? | T2D | ND | P | 1.22 | | | | | <0.05 |
| ▲Masud et al.[9] | Quantitative | UK | | | | | | | | | 1088 (CAD) | ? | A | Higher BMI/ cholesterol | 0.020 |
| | Meta-analysis [1,3-7,9-32] | USA, Finland, Japan, Denmark, Sweden, Italy, Germany, France, Mexican-American, China, Korea, UK, Australia | | | | | | | | | 19136 | ? | A | Higher BMI (d=0.07) | 0.019 |
| | Meta-analysis [1,3-6,9,10,12-14,20,21,23,24,26,27,32] | USA, Finland, Denmark, Italy, Germany, France, Mexican-American, Australia, | | | | | | | | | 8365 (Obese (BMI≥27)) | ? | A | Higher BMI (d=0.11) | 0.0006 |
| ▲Tönjes et al.[33] | Meta-analysis [1-3,6,10,13,17,19,21,22,25,32,34-54] | Scandinavia, Canada, Germany, Austria, Finland, Sweden, Israel, Norway Denmark, Israel, Spain, Italy, USA, France, The Netherlands, UK, Uruguay | | | | | | | | | 18590 (Caucasian only) | ? | A | Higher BMI | 0.015 |

| Author | Study design | Population | # cases | # controls | Age cases | Age controls | Definition cases | Definition controls | Allele carriers | OR | # Quant | Mean age | Geno-type | Phenotype | *p* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Meta-analysis [34,36,48] | Canada, Germany, Austria, Finland, Norway, Denmark, Spain, France, Italy | | | | | | | | | 2492 (obese only (BMI>30)) | ±47 | PP | Higher HOMA-IR (d=0.148) /fasting glucose (d=0.127) | 0.0025/ 0.0033 |
| Scott et al.[55] | Case/control | Finland | 1161 | 1174 | 53[b] | 64 | T2D | ND | P | 1.30 | | | | | 0.0011 |
| | Case/control | Finland | 1215 | 1258 | 56[b] | 59 | T2D | ND | P | 1.08 | | | | | 0.33 |
| | Case/control | Finland | 2376 | 2432 | ±55 | ±62 | T2D | ND | P | 1.20 | | | | | $1.4*10^{-3}$ |
| | Meta-analysis | Finland, Sweden, US, Poland, UK | 10829 | 12622 | ? | ? | T2D | ND | P | 1.14 | | | | | $1.7*10^{-6}$ |
| Saxena et al.[56] | Case/control | Finland, Sweden | 1464 | 1467 | 58[b] | 58 | T2D | ND | P | 1.02 | | | | | 0.83 |
| | Case/control | Sweden, US, Poland | 5065 | 5785 | ±55 | ±60 | T2D | ND | P | 1.11 | | | | | $6.2*10^{-3}$ |
| | Case/control | Finland, Sweden, US, Poland | 6529 | 7252 | ±55 | ±60 | T2D | ND | P | 1.09 | | | | | 0.019 |
| | Meta-analysis | Finland, Sweden, US, Poland, UK | 10829 | 12622 | ? | ? | T2D | ND | P | 1.14 | | | | | $1.7*10^{-6}$ |
| Zeggini et al.[57] | Case/control | UK | 1924 | 2938 | 50[b] | ? | T2D | ? | P | 1.23 | | | | | $1.3*10^{-3}$ |
| | Meta-analysis | Finland, Sweden, US, Poland, UK | 10829 | 12622 | ? | ? | T2D | ND | P | 1.14 | | | | | $1.7*10^{-6}$ |

| Author | Study design | Population | # cases | # controls | Age cases | Age controls | Definition cases | Definition controls | Allele carriers | OR | # Quant | Mean age | Geno-type | Phenotype | *p* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| ▲Pihlajamaki et al.[58] | Quantitative | Finland | | | | | | | | | 311 | 0 | A | Higher ponderal index at birth/birth weight | 0.007/ 0.094 |
| | Quantitative | Finland | | | | | | | | | 311 | 7 | AA | Higher weight | 0.045 |
| | Quantitative | Finland | | | | | | | | | 311 | 20 | | Weight | NS |
| | Quantitative | Finland | | | | | | | | | 311 | 41 | A | Higher waist circumference/ weight/BMI | 0.04/ NS |
| ◆Pfab et al.[59] | Quantitative | Germany | | | | | | | | | 1930 | 0 | | Birth weight/ ponderal index/length at birth | NS |
| ◆Labayen et al.[60] | Quantitative | Spain | | | | | | | | | 273 | 0 | | Birth weight | NS |
| | Quantitative | Spain | | | | | | | | | 273 | 15 | | Obesity parameters | NS |
| Souren et al. (This study) | Quantitative (twin study) | Belgium | | | | | | | | | 596 | 0 | A | Higher birth weight | 0.04 |
| | Quantitative (twin study) | Belgium | | | | | | | | | 600 | 25 | XA | Higher WHR | 0.02 |
| | Quantitative (twin study) | Belgium | | | | | | | | | ±600 | 25 | | Glucose/ insulin/lipid parameters | NS |

[a]Original association study. [b]Age at diagnosis. CAD = coronary artery disease, IGT = impaired glucose tolerance, ND = non-diabetic, NS = non-significant, OR = odds ratio, Quant = quantitative trait analysis, T2D = type 2 diabetes.

**Table S6.** Summary of published studies about the association between the *IL6* (-174G>C) SNP and T2D and/or related traits.

| Author | Study design | Population | # cases | # controls | Age cases | Age controls | Definition cases | Definition controls | Allele carriers | OR | # Quant | Mean age | Geno-type | Phenotype | *p* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Fishman et al.[61a] | Quantitative | UK | | | | | | | | | 102 | 40-75 | G | Higher IL-6 levels | 0.02 |
| Brull et al.[62] | Quantitative | UK | | | | | | | | | 127 | 63 | CC | Higher IL-6 levels 6 h after coronary artery bypass surgery | 0.04 |
| Burzotta et al.[63] | Quantitative | Italy | | | | | | | | | 111 | 61 | GG | Higher AUC IL-6 levels after coronary artery bypass surgery | 0.042 |
| Schlüter et al.[64] | Quantitative | Germany | | | | | | | | | 45 | ? | | IL-6 levels during sepsis | NS |
| Bonafe et al.[65] | Quantitative | Italy | | | | | | | | | 42 (men) | 71 | GG | Higher IL-6 levels | 0.043 |
| | Quantitative | Italy | | | | | | | | | 28 (men) | 91 | GG | Higher IL-6 levels | 0.024 |
| | Quantitative | Italy | | | | | | | | | 18 (men) | 101 | GG | Higher IL-6 levels | 0.038 |
| | Quantitative | Italy | | | | | | | | | 47 (women) | 71 | GG | Higher IL-6 levels | NS |
| | Quantitative | Italy | | | | | | | | | 82 (women) | 91 | GG | Higher IL-6 levels | 0.069 |
| | Quantitative | Italy | | | | | | | | | 52 (women) | 101 | GG | Higher IL-6 levels | NS |
| ▲ & ▼ Fernandez-Real et al.[66] | Quantitative | Spain | | | | | | | | | 32 | 37 | G | Higher IL-6 /insulin/ triglycerides/ FFA/lower HDL-cholesterol | 0.09/ 0.02/ 0.012/ 0.012/ 0.08 |

| Author | Study design | Population | # cases | # controls | Age cases | Age controls | Definition cases | Definition controls | Allele carriers | OR | # Quant | Mean age | Geno-type | Phenotype | *p* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Bennermo et al.[67] | Quantitative | Sweden | | | | | | | | | 38 | ±58 | GG | Higher IL-6 levels at 6, 8 & 10 hours after vaccination | <0.01 |
| Endler et al.[68] | Quantitative | Austria | | | | | | | | | 76 | 19-40 | | IL-6 levels before and after LPS infusion | NS |
| Nauck et al.[69] | Case/control | Germany | 2575 | 729 | 64 | 58 | CAD patients | Non CAD or MI | C | 0.9 | | | | | NS |
| | Case/control | Germany | 1365 | 729 | 63 | 58 | MI patients | Non CAD or MI | C | 0.98 | | | | | NS |
| | Quantitative | Germany | | | | | | | | | 940 | ? | | IL-6 levels | NS |
| ◆Jerrard-Dunne et al.[70] | Quantitative | Germany | | | | | | | | | 1000 | 50-65 | | BMI/lipid parameters | NS |
| | Quantitative | Germany | | | | | | | | | 500 | 50-65 | CC | Higher IL-6 levels | <0.05 |
| Unfried et al.[71] | Quantitative | Austria | | | | | | | | | 124 (women) | 56 | | IL-6 levels | NS |
| | Quantitative | Austria | | | | | | | | | 161 (women) | 32 | | IL-6 levels | NS |
| Jones et al.[72] | Quantitative | UK | | | | | | | | | 466 (AAA patients) | 69 | CC | Higher IL-6 levels | 0.047 |
| ◆Basso et al.[73] | Case/control | UK | 498 | 1108 | 56 | 56 | CHD | Non CHD | C | 1.01 | | | | | NS |
| | Quantitative | UK | | | | | | | | | 281 | ±56 | | IL-6 levels | NS |
| | Quantitative | UK | | | | | | | | | 1108 | ±56 | | BMI/ triglycerides /total, LDL & HDL-cholesterol | NS |
| | Quantitative | UK | | | | | | | | | 187 (CHD) | ±56 | | IL-6 levels | NS |

| Author | Study design | Population | # cases | # controls | Age cases | Age controls | Definition cases | Definition controls | Allele carriers | OR | # Quant | Mean age | Geno-type | Phenotype | *p* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Quantitative | UK | | | | | | | | | 498 (CHD patients) | ±56 | | BMI/ triglycerides/ total, LDL & HDL-cholesterol | NS |
| ◆Jenny et al.[74] | Quantitative | US | | | | | | | | | 1424 | ±72 | | IL-6 levels /obesity/lipid parameters | NS |
| Kilpinen et al.[75] | Quantitative | Finland | | | | | | | | | 92 (neonates) | 0 | CC | Higher IL-6 levels | <0.03 |
| | Quantitative | Finland | | | | | | | | | 450 (adults) | ? | | IL-6 levels | NS |
| Roth et al.[76] | Quantitative | US | | | | | | | | | 242 | 21-92 | G | Higher fat free mass (in men only) | 0.02 |
| ◆Kubaszek et al.[77] | Quantitative | Finland | | | | | | | | | 72 | 52 | | IL-6 levels | NS |
| | Quantitative | Finland | | | | | | | | | 124 | 52 | G | Obesity/higher energy expenditure/ higher whole body glucose uptake/higher oxidative & non-oxidative glucose disposal | NS/ <0.05/ 0.016/ 0.013/ 0.016 |
| ▲Berthier et al.[78] | Quantitative | Canada | | | | | | | | | 270 (men) | 43 | C | Higher weight/BMI/fat mass/waist /subcutaneous adipose tissue/FFA | 0.053/ 0.06/ 0.07/ 0.02/ 0.051/ 0.07 |
| | Quantitative | Canada | | | | | | | | | 270 (men) | 43 | | Glucose/insulin parameters | NS |

| Author | Study design | Population | # cases | # controls | Age cases | Age controls | Definition cases | Definition controls | Allele carriers | OR | # Quant | Mean age | Geno-type | Phenotype | *p* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Vozarova et al.[79] | Case/control | Spain | 211 | 118 | 53[b] | 57 | T2D | ND | G | 1.60 | | | | | 0.028 |
| | Case/control | US (Native Americans) | 143 | 145 | 17[b] | 64 | T2D | ND | G | 9.39 | | | | | 0.019 |
| | Family based | US (Native Americans) | 65 | 110 | 30[b] | 33 | T2D | ND | GG | 2.23 | | | | | NS |
| ▲Illig et al.[80] | Case/control | Germany | 230 | 239 | 65 | 65 | T2D | ND | G | 1.51 | | | | | 0.0096 |
| | Case/control | Germany | 235 | 239 | 66 | 65 | IGT | ND | | | | | | | NS |
| | Quantitative | Germany | | | | | | | | | 650 (all) | 65 | C | Higher waist/BMI | 0.059/ 0.039 |
| | | | | | | | | | | | 650 (all) | 65 | | IL-6/lipid /insulin/ glucose parameters | NS |
| ▲ & ▼ Hamid et al.[81] | Case/control | Denmark | 1389 | 4401 | 51[b] | 45 | T2D | ND | GG | 1.05 | | | | | NS |
| | Case/control | Denmark | 1389 | 1464 | 51[b] | 51 | T2D | ND | GG | ? | | | | | NS |
| | Case/control | Denmark | 1009 | 2581 | ? | ? | Obese (BMI>30) | Non-obese (BMI≤25) | G | 1.?? | | | | | 0.008 |
| | Case/control | Denmark | 1524 | 1661 | ? | ? | Impaired glucose regulation | Healthy[c] | GG | ? | | | | | NS |
| | Case/control | Denmark | 1532 | 1661 | ? | ? | Insulin resistance | Healthy[c] | G | 1.?? | | | | | 0.02 |
| | Case/control | Denmark | 2423 | 1661 | ? | ? | Hyperten-sion | Healthy[c] | G | 1.?? | | | | | 0.05 |
| | Case/control | Denmark | 2566 | 1661 | ? | ? | Obesity (BMI>30 or WHR>0.90 (men) WHR>0.85 (women) | Healthy[c] | G | ? | | | | | NS |

| Author | Study design | Population | # cases | # controls | Age cases | Age controls | Definition cases | Definition controls | Allele carriers | OR | # Quant | Mean age | Geno-type | Phenotype | *p* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Case/control | Denmark | 1543 | 1661 | ? | ? | Dyslipi-daemia | Healthy[c] | G | 1.?? | | | | | 0.007 |
| | Case/control | Denmark | 168 | 1661 | ? | ? | Micro-albuminuria | Healthy [c] | G | 1.?? | | | | | 0.07 |
| | Case/control | Denmark | 4337 | 1661 | ? | ? | One or more components of the metabolic syndrome | Healthy[c] | G | 1.?? | | | | | 0.07 |
| | Case/control | Denmark | 1277 | 1661 | ? | ? | Metabolic syndrome | Healthy[c] | G | 1.?? | | | | | 0.03 |
| | Quantitative | Denmark | | | | | | | | | 4401 | 45 | G | Higher BMI/insulin AUC | 0.02/ 0.02 |
| | | | | | | | | | | | 4401 | 45 | | Glucose/lipid parameters | NS |
| ▲Yang et al.[82] | Quantitative | Sweden | | | | | | | | | 85 | 48 | G | Higher triglycerides/ insulin/ HOMA-IR/lower adiponectin levels | 0.02/ 0.03/ 0.03/ 0.02 |
| | Quantitative | Sweden | | | | | | | | | 85 | 48 | | Obesity/IL-6 | NS |
| | Quantitative | ? | | | | | | | | | 215 | ? | G | Higher insulin/ HOMA-IR | 0.07/ 0.06 |
| | Quantitative | ? | | | | | | | | | 44 | ? | G | Higher adipose tissue *IL-6* expression | 0.037 |
| ▲Barbieri et al.[83] | Quantitative | Italy | | | | | | | | | 429 | 72 | G | Higher IL-6/ cholesterol levels | 0.03/ 0.048 |

| Author | Study design | Population | # cases | # controls | Age cases | Age controls | Definition cases | Definition controls | Allele carriers | OR | # Quant | Mean age | Geno-type | Phenotype | *p* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Quantitative | Italy | | | | | | | | | 429 | 72 | | Obesity/ glucose/ insulin parameters | NS |
| Bennet et al.[84] | Case/control | Sweden | 812 | 1013 | 59 | 59 | MI (men) | Non MI (men) | XC | 1.2 | | | | | NS |
| | Case/control | Sweden | 345 | 487 | 63 | 63 | MI (women) | Non MI (women) | XC | 1.2 | | | | | NS |
| | Quantitative | Sweden | | | | | | | | | 607 (MI men) | 59 | | IL-6/insulin levels | NS |
| | Quantitative | Sweden | | | | | | | | | 783 (men) | 59 | G | Higher insulin levels/IL-6 | 0.01/ NS |
| | Quantitative | Sweden | | | | | | | | | 308 (MI women) | 63 | | IL-6/insulin levels | NS |
| | Quantitative | Sweden | | | | | | | | | 433 (women) | 63 | | IL-6/insulin levels | NS |
| ▼Cardellini et al.[85] | Quantitative | Italy | | | | | | | | | 275 | 38 | GG | Higher IL-6 /2-h glucose/ fasting insulin/ fibrinogen/ white blood cell count/ lower glucose disposal | 0.02/ 0.09/ 0.09/ 0.02/ 0.03/ 0.004 |
| | Quantitative | Italy | | | | | | | | | 77 (obese) | 39 | GG | Higher fasting insulin/ HOMA/IL-6 mRNA levels/ lower cholesterol/ triglycerides | 0.04/ 0.07/ 0.04/ 0.007 /0.04 |

| Author | Study design | Population | # cases | # controls | Age cases | Age controls | Definition cases | Definition controls | Allele carriers | OR | # Quant | Mean age | Geno-type | Phenotype | *p* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| ▲Stephens et al.[86] | Quantitative | UK | | | | | | | | | 571 (T2D) | ±67 | C | Higher BMI/lipid parameters | 0.05/ NS |
| | Quantitative | UK | | | | | | | | | 2652 | 56 | | BMI | NS |
| | Case/control | UK | 289 | 282 | 64 | 69 | T2D & obese (BMI>29.1) | T2D & non-obese (BMI<29.1) | C | 1.?? | | | | | 0.008 |
| | Case/control | UK | 1344 | 1308 | ? | ? | Obese (BMI>26.1) | Non-obese (BMI<26.1) | | ? | | | | | NS |
| | Case/control | UK | 493 | 2159 | ? | ? | Obese (BMI>29.1) | Non-obese (BMI<29.1) | | ? | | | | | NS |
| | Case/control | UK | 571 | 2652 | 68 | 56 | T2D | ND | C | 0.?? | | | | | <0.00 001 |
| ◆Ferrari et al.[87] | Quantitative | US | | | | | | | | | 816 (women) | 60 | | BMI | NS |
| | Quantitative | US | | | | | | | | | 745 (men) | 60 | | BMI | NS |
| ▼Chapman et al.[88] | Quantitative | Australia | | | | | | | | | 1109 | 53 | | BMI/WHR/ HDL- & LDL-cholesterol | NS |
| | Quantitative | Australia | | | | | | | | | 1109 | 53 | CC | Higher triglycerides | 0.03 |
| ▲Moffett et al.[89] | Quantitative | US | | | | | | | | | 3376 | 73 | GG | Higher BMI | 0.027 |
| ◆Jeng et al.[90] | Case/control | China | 207 | 217 | 52 | 48 | Hyperten-sive | Normo-tensive | G | 2.23 | | | | | 0.04 |
| | Quantitative | China | | | | | | | | | 217 (normo-tensive) | 48 | | BMI/ glucose/ lipid parameters | NS |
| | Quantitative | China | | | | | | | | | 207 (hyper-tensive) | 52 | | BMI/ glucose/ lipid parameters | NS |

| Author | Study design | Population | # cases | # controls | Age cases | Age controls | Definition cases | Definition controls | Allele carriers | OR | # Quant | Mean age | Geno-type | Phenotype | *p* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| ▲Eklund et al.[91] | Quantitative | Finland | | | | | | | | | 78 | 35-50 | CC | Higher fat mass/IL-6 levels | 0.05/ NS |
| ◆Garnero et al.[92] | Quantitative | France | | | | | | | | | 255 (women) | 40 | | BMI | NS |
| | Quantitative | France | | | | | | | | | 372 (women) | 64 | | BMI | NS |
| ◆Möhlig et al.[93] | Case/control | Germany | 188 | 376 | 56 | 56 | T2D | ND | | | | | | | NS |
| | Quantitative | Germany | | | | | | | | | 564(all) | 56 | | BMI/IL-6 levels | NS |
| Tsiavou et al.[94] | Case/control | Greece | 32 | 39 | 51 | 44 | T2D | ND | | | | | | | NS |
| ▲Wernstedt et al.[95] | Quantitative | Sweden | | | | | | | | | 485 (hyper-tensive) | 57 | C | Higher BMI/CRP | 0.003 /0.06 |
| | Quantitative | Sweden | | | | | | | | | 485 (hyper-tensive) | 57 | | IL-6 levels /glucose/ insulin/lipid parameters | NS |
| | Quantitative | Sweden | | | | | | | | | 333 (males) | | C | Higher leptin levels | 0.002 |
| | | | | | | | | | | | 120 (females) | | C | Higher leptin levels | 0.033 |
| | Case/control | Sweden | 347 | 124 | ? | ? | Obese (BMI>25) | Non-obese (BMI<25) | GC CC | 1.71 2.27 | | | | | 0.013 |
| | Quantitative | Sweden | | | | | | | | | 74 | 38 | C | Higher BMI/leptin | 0.03/ 0.09 |
| ◆Lieb et al.[96] | Quantitative | Germany | | | | | | | | | 743 (MI) | 58 | | BMI/lipid parameters | NS |
| | Family based | Germany | | | | | | | | | 579 (MI) | 56 | | Il-6 levels/ BMI/lipid parameters | NS |
| | Quantitative | Germany | | | | | | | | | 1023 | 46 | | BMI/lipid parameters | NS |
| | Case/control | Germany | 1322 | 1023 | ±57 | 52 | MI | Non-MI | ? | ? | | | | | NS |

| Author | Study design | Population | # cases | # controls | Age cases | Age controls | Definition cases | Definition controls | Allele carriers | OR | # Quant | Mean age | Geno-type | Phenotype | *p* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| ▲ & ▼ Goyeneche a et al.[97] | Quantitative | Spain | | | | | | | | | 106 (obese) | 33 | C | Higher waist/ insulin/ HOMA-IR/lower adiponectin /leptin | 0.07/ 0.04/ 0.01/ 0.01/ 0.03 |
| | Case/control | Spain | 58 | 48 | 33 | 33 | High risk of developing metabolic complications | Low risk of developing metabolic complications | C | 5.2 | | | | | 0.003 |
| ◆Testa et al.[98] | Case/control | Italy | 238 | 255 | 61 | 60 | T2D | ND | | | | | | | NS |
| | Quantitative | Italy | | | | | | | | | 238 (T2D) | 61 | | Il-6/ BMI/ glucose/ insulin/ lipid parameters | NS |
| | Quantitative | Italy | | | | | | | | | 255 | 60 | | Il-6/ BMI/ glucose/ insulin/ lipid parameters | NS |
| Qi et al.[99] | Case/control | US | 1532 | 2137 | ? | ? | T2D (women) | ND (women) | G | 1.02 | | | | | NS |
| | Case/control | US | 910 | 911 | ? | ? | T2D (men) | ND (men) | G | 1.03 | | | | | NS |
| | Quantitative | US | | | | | | | | | 718 (women) | ? | | IL-6 levels | NS |
| | Meta-analysis [77,79-81,93,94,98-100] | Spain, Finland, Germany, Greece, Denmark, Italy, US, Sweden | 5383 | 12069 | ? | ? | T2D | ND | GG | 1.06 | | | | | NS |

| Author | Study design | Population | # cases | # controls | Age cases | Age controls | Definition cases | Definition controls | Allele carriers | # OR | Quant | Mean age | Geno-type | Phenotype | *p* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| ◆Vaxillaire et al.[101] | Case/control | France | 187 | 2919 | ? | ? | T2D | ND | C | 0.70 (HR) | | | | | 0.002 |
| | Quantitative | France | | | | | | | | | 2919 | ? | | BMI/glucose/ insulin parameters | NS |
| ▲ & ▼ Stephens et al.[102] | Quantitative | UK | | | | | | | | | 571 (T2D) | 67 | C | Higher BMI/IL-6 levels | 0.03/ 0.05 |
| | Case/control | UK | 354 | 217 | 67 | 67 | Metabolic syndrome | No metabolic syndrome | C | 1.?? | | | | | 0.002 |
| | Case/control | UK | ? | ? | 67 | 67 | Obese (BMI>30) | Non-obese (BMI≤30) | C | 1.?? | | | | | 0.001 |
| Walston et al.[103] | Quantitative | European-American | | | | | | | | | 4546 | 72 | C | Higher IL-6/CRP levels | 0.04/ 0.0008 |
| | Quantitative | African-American | | | | | | | | | 839 | 72 | | IL-6/CRP levels | NS |
| ▲Halverstadt et al.[104] | Quantitative | US | | | | | | | | | 65 | 58 | | Obesity/lipid parameters | NS |
| | Quantitative | US | | | | | | | | | 65 | 58 | CC | Higher HDL-cholesterol increase after exercise training | 0.003 |
| ▲Willer et al.[105] | Case/control | Finland | 1130 | 946 | ±54[b] | ±65 | T2D | ND | GG | 1.25 | | | | | 0.038 |
| | Quantitative | Finland | | | | | | | | | 375 | 70 | G | Higher disposition index/glucose effectiveness /lower FFA/HDL | 0.008/ 0.01/ 0.001/ 0.009 |

| Author | Study design | Population | # cases | # controls | Age cases | Age controls | Definition cases | Definition controls | Allele carriers | OR | # Quant | Mean age | Geno-type | Phenotype | *p* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| ▲Shen et al.[106] | Quantitative | US | | | | | | | | | 720 | 48 | G | Higher IL-6/ triglycerides/ VLDL/large VLDL/ smaller HDL-size | 0.029/ 0.025/ 0.04/ 0.02/ 0.03 |
| Herbert et al.[107] | Family based | US | | | | | | | | | 450 | 51 | C | Higher fasting glucose | 0.008 |
| ▼Sie et al.[108] | Quantitative | The Netherlands | | | | | | | | | 6434 | 70 | GC vs CC | Higher BMI/lipid parameters | 0.04/ NS |
| | Quantitative | The Netherlands | | | | | | | | | 5924 | 70 | C | Higher CRP levels | <0.01 |
| | Quantitative | The Netherlands | | | | | | | | | 641 | ? | | IL-6 levels | NS |
| | Meta analysis [69,73,84,96,108-111] | UK, France, Germany, Sweden, Italy, The Netherlands | 6927 | 12871 | ? | ? | CHD | Non CHD | XC | 1.12 | | | | | NS |
| ◆Qi et al.[112] | Quantitative | US | | | | | | | | | 980 (men) | 56 | | BMI | NS |
| | Quantitative | US | | | | | | | | | 2255 (women) | 44 | | BMI | NS |
| | Meta-analysis [76-78,80-82,85,87-93,96,108,113] | Finland, Canada, US, Germany, Denmark, Sweden, The Netherlands, Italy, France, China, Australia | | | | | | | | | 26944 | ? | | BMI | NS |

| Author | Study design | Population | # cases | # controls | Age cases | Age controls | Definition cases | Definition controls | Allele carriers | OR | # Quant | Mean age | Geno-type | Phenotype | *p* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Huth et al.[114] | Meta-analysis [79-81,86,93-96,114] | Finland, Sweden, Denmark, UK, Germany, Spain, Greece, US | 4746 | 16230 | ? | ? | T2D | ND | C | 0.91 (adjusted for BMI) | | | | | 0.037 |
| Souren et al. (This study) | Quantitative (Twin study) | Belgium | | | | | | | | | 564 | 25 | CC | Higher HDL cholesterol | 0.008 |
| | Quantitative (Twin study) | Belgium | | | | | | | | | ±580 | 25 | | Birth weight/obesity/ glucose/insulin/ other lipid parameters | NS |
| | Quantitative (Twin study) | Belgium | | | | | | | | | 272 (men only) | 25 | CC | Higher NEFA | 0.005 |
| | Quantitative (Twin study) | Belgium | | | | | | | | | 308 (women only) | 25 | C | Higher S4SF/lower triacylglycerol | 0.002/ 0.04 |

[a]Original association study. [b]Age at diagnosis. [c]Subjects having no components of the metabolic syndrome. AAA = abdominal aortic aneurysm, CAD = coronary artery disease, CHD = coronary heart disease, CRP = C-reactive protein, HR = hazard ratio, IGT = impaired glucose tolerance, MI = myocardial infarction, ND = non-diabetic, NS = non-significant, OR = odds ratio, Quant = quantitative trait analysis, T2D = type 2 diabetes.

**Table S7.** Summary of published studies about the association between the *GAD2* (-243A>G) SNP and type 2 diabetes and/or related traits.

| Author | Study design | Population | # cases | # controls | Age cases | Age controls | Definition cases | Definition controls | Allele carriers | OR | # Quant | Mean age | Geno-type | Phenotype | *p* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| ▼Boutin et al.[115a] | Case/control | France | 348 | 371 | 46 | 58 | Obese (mean BMI=47) | Non-obese (mean BMI=23) | G | 1.32 | | | | | 0.04 |
| | Case/control | France | 224 | 243 | 47 | 44 | Obese (mean BMI=47) | Non-obese (mean BMI=23) | G | 1.25 | | | | | 0.17 |
| | Case/control | France | 572 | 614 | ±46 | ±50 | Obese (mean BMI=47) | Non-obese (mean BMI=23) | G | 1.30 | | | | | 0.014 |
| | Family based | France | | | | | | | | | 612 | | G | Higher obesity rate | 0.06 |
| | Quantitative | France | | | | | | | | | 464 (Obese) | | GG | Higher hunger scores & disinhibition scores | 0.007 / 0.028 |
| | Functional | | | | | | | | | | | | GG | Higher transcriptional activity | <0.0001 |
| Swarbrick et al.[116] | Family based | Germany | | | | | | | | | 2359 | | | Obesity | NS |
| | Case/control | US | 302 | 427 | 50 | 52 | Obese (mean BMI=48) | Non-obese (mean BMI=23) | G | 1.11 | | | | | NS |
| | Case/control | Canada | 378 | 759 | 46 | 45 | Obese (mean BMI=48) | Non-obese (mean BMI=20) | G | 0.90 | | | | | NS |
| | Case/control | US, Canada | 680 | 1186 | ±48 | ±48 | Obese (mean BMI=48) | Non-obese (mean BMI=20) | G | 0.99 | | | | | NS |
| | Meta analysis [115,116] | France, US, Canada | 1252 | 1800 | ? | ? | Obese | Non-obese | G | 1.11 | | | | | NS |
| | Functional | | | | | | | | | | | | G | Transcriptional activity | NS |

| Author | Study design | Population | # cases | # controls | Age cases | Age controls | Definition cases | Definition controls | Allele carriers | OR | # Quant | Mean age | Geno-type | Phenotype | *p* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| ◆Meyre et al.[117] | Case/control | France | 477 | 614 | ±10 | ±50 | Obese | Non-obese | G | 1.25 | | | | | 0.04 |
| | Case/control Quantitative | France France | 477 | 196 | ±10 | 15 | Obese | Non-obese | G | 1.27 | | | | | 0.10 |
| | | | | | | | | | | | 635 (obese) | 11 | G | Lower birth weight/birth height/lower AUC of BMI between 0 &8 year | 0.009/ 0.013/ 0.03 |
| | | | | | | | | | | | | | | Glucose/ insulin parameters | NS |
| | Family based | France | | | | | | | | | 1205 | | G | Obesity/birth weight/ insulinogenic index | NS |
| | Quantitative | France | | | | | | | | | 432 (obese) | 11 | XG | Higher rate of abnormal binge eating behaviour | 0.04 |
| ▲Groves et al.[118] | Family based | UK | | | | | | | | | 1215 | ? | G | Lower BMI | 0.025 |
| ▲Boesgaard et al.[119] | Quantitative | Denmark | | | | | | | | | 5857 | 46 | G | Lower BMI/fasting glucose/30 min glucose OGTT related | 0.01/ 0.008/ 0.04 |
| | Case/control | Denmark | 968 | 2582 | ±46 | ±46 | Obese (BMI≥30) | Non-obese (BMI<25) | G | 0.86 | | | | | 0.03 |
| | Case/control | Denmark | 2307 | 2582 | ±46 | ±46 | Moderately obese (25≤BMI≤30) | Non-obese (BMI<25) | G | 0.96 | | | | | NS |

| Author | Study design | Population | # cases | # controls | Age cases | Age controls | Definition cases | Definition controls | Allele carriers | OR | # Quant | Mean age | Geno-type | Phenotype | *p* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| ◆Hunt et al.[120] | Case/control | US (Utah) | 855 | 130 | ? | ? | Obese (mean BMI=48) | Non-obese (mean BMI=22) | | | | | | | NS |
| | Quantitative | US (Utah) | | | | | | | | | ? | ? | | Glucose/insulin parameters | NS |
| ▼Witchel et al.[121] | Quantitative | US (Pennsylvania) | | | | | | | | | 37 | 8 | G | Higher BMI | 0.0043 |
| | | | | | | | | | | | 37 | 17 | G | Higher BMI | 0.0046 |
| Souren et al. (This study) | Quantitative (Twin study) | Belgium | | | | | | | | | 587 | 25 | AG | Lower fasting insulin | 0.01 |
| | Quantitative (Twin study) | Belgium | | | | | | | | | ±600 | 25 | | Birth weight/obesity/ glucose/lipid parameters | NS |
| | Quantitative (Twin study) | Belgium | | | | | | | | | 277 (men only) | 25 | G | Lower lean body mass/fasting insulin | 0.004/ 0.004 |

[a]Original association study. AUC = area under the curve, NS = non-significant, OGTT = oral glucose tolerance test, OR = odds ratio, Quant = quantitative trait analysis.

**Table S8**. Summary of published studies about the association between the *PYY* (R72T) SNP and type 2 diabetes and/or related traits.

| Author | Study design | Population | # cases | # controls | Age cases | Age controls | Definition cases | Definition controls | Allele carriers | OR | # Quant | Mean age | Geno-type | Phenotype | *p* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| ◆Hung et al.[122a] | Quantitative | UK | | | | | | | | | 952 | 40-65 | | Obesity/glucose /insulin/lipid parameters | NS |
| ▼Torekov et al.[123] | Case/control | Denmark | 1326 | 4639 | 52[b] | 46 | T2D | ND | RR | 1.19 | | | | | 0.005 |
| | Case/control | Denmark | 2352 | 2635 | 47 | 45 | 25≤BMI<30 | BMI<25 | RR | 1.15 | | | | | 0.018 |
| | Case/control | Denmark | 1035 | 2635 | 48 | 45 | BMI≥30 | BMI<25 | RR | ? | | | | | NS |
| | Case/control | Denmark | 3387 | 2635 | 47 | 45 | BMI≥25 | BMI<25 | RR | 1.14 | | | | | 0.019 |
| | Quantitative | Denmark | | | | | | | | | 6022 | 46 | RR | Higher glucose 2h after OGTT/ glucose AUC/lower insulinogenic index | 0.03/ 0.03/ 0.01 |
| ◆Lavebratt et al.[124] | Case/control | Sweden | 129 | 133 | 48 | 41 | Obese (25≤BMI<35) | Non-obese (BMI<25) | | | | | | | NS |
| | Case/control | Sweden | 226 | 133 | 45 | 41 | Morbidly obese (BMI≥35) | Non-obese (BMI<25) | | | | | | | NS |
| | Quantitative | Sweden | | | | | | | | | 458 | 45 | | Obesity/glucose /insulin/lipid parameters | NS |
| Ahituv et al.[125] | Case/control | Canada | 374 | 373 | ? | ? | Extremely obese (BMI>36) | Non-obese (mean BMI=19.5) | RT (hetero zygosit effect) | 0.?? | | | | | 0.02 |
| Siddiq et al.[126] | Case/control | France | 460 | 957 | ? | ±40 | Obese children | Non-obese & ND | T | 1.10 | | | | | NS |
| | Case/control | France | 482 | 957 | ? | ±40 | Morbidly obese adults | Non-obese & ND | T | 1.08 | | | | | NS |
| | Quantitative | France | | | | | | | | | 662 | Chil-dren | | BMI/WHR | NS |
| | Quantitative | France | | | | | | | | | 557 | Adults (obese) | | BMI/WHR | NS |

| Author | Study design | Population | # cases | # controls | Age cases | Age controls | Definition cases | Definition controls | Allele carriers | OR | # Quant | Mean age | Geno-type | Phenotype | *p* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Campbell et al.[127] | Case/control | European American, Poland | 886 | 439 | 56 | 56 | Obese men (mean BMI=34) | Non-obese men (mean BMI=22) | T | 1.00 | | | | | NS |
| | Case/control | European American, Poland | 1032 | 516 | 58 | 57 | Obese women (mean BMI=36) | Non-obese women (mean BMI=21) | T | 1.04 | | | | | NS |
| | Case/control | European American, Poland | 1918 | 955 | ±57 | ±57 | Obese (mean BMI=35) | Non-obese (mean BMI=22) | T | 1.02 | | | | | NS |
| Souren et al. (This study) | Quantitative (Twin study) | Belgium | | | | | | | | | ±600 | 25 | | Birth weight/obesity/ glucose/insulin/ lipid parameters | NS |
| | Quantitative (Twin study) | Belgium | | | | | | | | | 270 (women only | 25 | TT | Higher fasting glucose | 0.003 |

[a]Original association study. [b]Age at diagnosis. AUC = area under the curve, T2D = type 2 diabetes, ND = non-diabetic, NS = non-significant, OGTT = oral glucose tolerance test, OR = odds ratio, Quant = quantitative trait analysis.

**Table S9.** Summary of published studies about the association between the *ENPP1* (K121Q) SNP and type 2 diabetes and/or related traits.

| Author | Study design | Population | # cases | # controls | Age cases | Age controls | Definition cases | Definition controls | Allele carriers | OR | # Quant | Mean age | Geno-type | Phenotype | *p* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| ▼Pizzuti et al.[128a] | Quantitative | Sicily | | | | | | | | | 121 | 37 | XQ | Higher glucose/ insulin/insulin sensitivity/ cholesterol | 0.0002/ <0.0001 /0.005/ 0.04 |
| | Case/control | Sicily | ? | ? | ? | ? | High insulin levels | Low insulin levels | Q | 2.99 | | | | | <0.001 |
| | | | | | | | | | | | 10 | | KQ vs KK | Reduced insulin receptor tyrosine kinase activity in cultured skin fibroblasts | <0.01 |
| ◆Gu et al.[129] | Quantitative | Finland, Sweden | | | | | | | | | 147 | 55 | | Obesity/ glucose/insulin/ lipid parameters | NS |
| | Quantitative | Finland, Sweden | | | | | | | | | 383 (ND T2D relatives) | 45 | QK vs KK | Higher glucose | <0.05 |
| | Quantitative | Finland, Sweden | | | | | | | | | 392 (T2D) | 58 | QK vs KK | Higher glucose | <0.001 |
| | Quantitative (sib pairs) | Finland, Sweden | | | | | | | | | 52 | 35 | QK vs KK | Higher BMI/ glucose/insulin | 0.07/ $8*10^{-5}$/ 0.02 |
| | Quantitative (sib pairs) | Finland, Sweden | | | | | | | | | 94 (T2D) | 58 | QK vs KK | Higher glucose/insulin | 0.02/ 0.08 |
| Rasmussen et al.[130] | Case/control | Denmark | 404 | 237 | 55 | 52 | T2D | ND | | | | | | | NS |
| | Quantitative | Denmark | | | | | | | | | 237 | 52 | | BMI/glucose/ insulin parameters | NS[c] |
| | Quantitative | Denmark | | | | | | | | | 226 (ND offspring T2D) | 39 | | BMI/glucose/ insulin parameters | NS[c] |

| Author | Study design | Population | # cases | # controls | Age cases | Age controls | Definition cases | Definition controls | Allele carriers | OR | # Quant | Mean age | Geno-type | Phenotype | *p* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Quantitative | Denmark | | | | | | | | | 356 | 25 | | BMI/glucose/ insulin | NS |
| Hegele et al.[131] | Case/control | Canada | 121 | 468 | ? | ? | T2D | ND | Q | 1.12 | | | | | NS |
| ▼Frittitta et al.[132] | Case/control | Sicily | 211 | 220 | 37 | 37 | Obese (BMI>29.9) | Non-obese (BMI≤29.9) | | | | | | | NS |
| | Quantitative | Sicily | | | | | | | | | 211 (Obese) | 37 | Q | Higher insulin during OGTT | <0.01 |
| | Quantitative | Sicily | | | | | | | | | 220 (Non-obese) | 37 | Q | Higher cholesterol/ insulin & glucose during OGTT | <0.05 |
| | Quantitative | Sicily | | | | | | | | | 431 (all) | 37 | Q | Higher insulin during OGTT | <0.01 |
| | Quantitative | Sicily | | | | | | | | | 131 | ±37 | Q | Lower insulin-sensitivity | <0.05 |
| ◆Kubaszek et al.[133] | Quantitative | Finland | | | | | | | | | 110 | 51 | Q | Higher glucose/ insulin/lower whole body glucose uptake/lower glucose disposal during clamp | 0.002/ 0.05/ 0.01/ 0.015 |
| | Quantitative | Finland | | | | | | | | | 110 | 51 | Q | BMI/lipid parameters | NS |
| | Quantitative | Finland | | | | | | | | | 295 | 44 | Q | Higher fasting insulin | 0.009 |
| | Quantitative | Finland | | | | | | | | | 295 | 44 | Q | BMI/lipid parameters | NS |
| ▼González-Sánchez et al.[134] | Quantitative | Spain | | | | | | | | | 293 | 48 | QK vs KK | Higher leptin/ triglycerides | 0.02/ 0.01 |
| | Quantitative | Spain | | | | | | | | | 293 | 48 | | BMI/glucose/ insulin | NS |

| Author | Study design | Population | # cases | # controls | Age cases | Age controls | Definition cases | Definition controls | Allele carriers | OR | # Quant | Mean age | Geno-type | Phenotype | *p* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Case/control | Spain | 91 | 202 | ±48 | ±48 | Obese (BMI≥30) | Non-obese (BMI<30) | | | | | | | NS |
| Barroso et al.[135] | Case/control | UK | 517 | 517 | 64 | 64 | T2D | ND | Q | 1.10 | | | | | NS |
| | Quantitative | | | | | | | | | | 1100 | ±54 | QQ | Higher BMI | <0.05 |
| Baratta et al [35] | Quantitative | Sicily | | | | | | | | | 338 | 38 | Q | Higher glucose/ insulin AUC/lower insulin sensitivity during OGTT | <0.05 |
| Hamaguchi et al.[136] | Case/control | Dominican Republic | 122 | 275 | 46 | 41 | Obese | Non-obese | Q | 1.?? | | | | | 0.088 |
| | Case/control | Dominican Republic | 55 | 275 | 54 | 41 | T2D (Non-obese) | Non-obese | Q | 1.56 | | | | | 0.037 |
| | Case/control | Dominican Republic | 303 | 275 | 53 | 41 | T2D (Obese) | Non-obese | Q | 1.27 | | | | | 0.043 |
| | Quantitative | Dominican Republic | | | | | | | | | 118 | 38 | Q | Higher immuno-reactive insulin/HOMA-β/ lower insulin sensitivity | <0.05 |
| Morrison et al.[137] | Quantitative | US (Black) | | | | | | | | | 481 | Chil-dren | | Insulin/glucose parameters | NS |
| | Quantitative | US (White) | | | | | | | | | 459 | Chil-dren | | Insulin/glucose parameters | NS |
| Meyre et al.[138] | Case/control | France | 526 | 548 | 10 | 55 | Obese (BMI=±28) | Non-obese (BMI=22) | Q | 1.45 | | | | | 0.001 |
| | | | | | | | | | QQ | 3.63 | | | | | 0.001 |
| | Case/control | France | 680 | 623 | 46 | 51 | Morbidly Obese (BMI=±47) | Non-obese (BMI=23) | Q | 1.31 | | | | | 0.01 |
| | | | | | | | | | QQ | 3.05 | | | | | 0.004 |
| | Case/control | France | 1206 | 1205 | ? | ? | Obese (all) | Non-obese (all) | Q | 1.37 | | | | | 0.00008 |
| | | | | | | | | | QQ | 3.29 | | | | | 0.00003 |

| Author | Study design | Population | # cases | # controls | Age cases | Age controls | Definition cases | Definition controls | Allele carriers | OR | # Quant | Mean age | Geno-type | Phenotype | *p* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | TDT | France | | | | | | | | | 184 families | | Q | Obesity | 0.01 |
| | Quantitative | France | | | | | | | | | 89 | 13 | Q including risk haplotype | Higher ENPP1 levels | 0.01 |
| | Case/control | France | 752 | 556 | 59 | 55 | T2D | ND | Q including risk haplotype | 1.44 | | | | | 0.005 |
| | Case/control | Austria | 503 | 758 | 56 | 52 | T2D | ND | Q including risk haplotype | 1.68 | | | | | 0.001 |
| | Case/control | France, Austria | 1255 | 1314 | ±57 | ±53 | T2D | ND | Q including risk haplotype | 1.56 | | | | | 0.00002 |
| ◆Abate et al.[139] | Quantitative | South Asians living in India | | | | | | | | | 456/ 223 (T2D) | 44/42 | | BMI/glucose/ lipid parameters | NS |
| | Quantitative | South Asians living in Dallas | | | | | | | | | 962/ 121 (T2D) | 43/55 | Q | Higher glucose (T2D)/BMI/lipid parameters | <0.05/ NS |
| | Quantitative | Caucasians living in Dallas | | | | | | | | | 717/ 141 (T2D) | 45/58 | | BMI/glucose/ lipid parameters | NS |
| | Quantitative | Caucasians living in Dallas | | | | | | | | | 152 | 29 | | BMI/insulin AUC/glucose/ lipid parameters | NS |
| | Quantitative | South Asians living in Dallas | | | | | | | | | 153 | 32 | | BMI/glucose/ lipid parameters | NS |
| | | | | | | | | | | | | | | Higher insulin AUC | <0.05 |

| Author | Study design | Population | # cases | # controls | Age cases | Age controls | Definition cases | Definition controls | Allele carriers | OR | # Quant | Mean age | Geno-type | Phenotype | *p* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Case/control | South Asians live in India | 223 | 456 | 42 | 44 | T2D | ND | Q | 1.62 | | | | | 0.01 |
| | Case/control | South Asians live in Dallas | 121 | 962 | 55 | 42 | T2D | ND | Q | 1.86 | | | | | 0.01 |
| | Case/control | Caucasians live in Dallas | 141 | 717 | 59 | 45 | T2D | ND | Q | 1.90 | | | | | 0.04 |
| | Meta analysis [129,130,133,136,139] | Finland, Sweden, Denmark, Dominican Republic, South Asia, US | 1733 | 3666 | ? | ? | T2D | ND | Q | 1.30 | | | | | 0.001 |
| Bacci et al.[140] | Case/control | Italy and US | 969 | 638 | 63 | 53 | T2D | ND | Q | 1.21 | | | | | 0.13 |
| | Meta analysis [128-130,133,136,139,140] | Italy, Finland, Sweden, Denmark, Dominican Republic, South Asia, US | 2834 | 4425 | ? | ? | T2D | ND | Q | 1.29 | | | | | 0.003 |
| | Case/control | Italy and US | 853 | 116 | ? | ? | T2D age of onset <65 | T2D age of onset ≥65 | Q | 2.26 | | | | | 0.006 |
| | Case/control | Italy and US | ? | ? | ? | ? | T2D & CAD (non-smokers) | T2D (non-smokers) | Q | 1.52 | | | | | 0.049 |
| | Case/control | Italy and US | 45 | 111 | ? | ? | T2D with age at myocardial infarction ≤50 | T2D with age at myocardial infarction >50 | Q | 2.98 | | | | | 0.007 |

| Author | Study design | Population | # cases | # controls | Age cases | Age controls | Definition cases | Definition controls | Allele carriers | OR | # Quant | Mean age | Geno-type | Phenotype | *p* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Weedon et al.[141] | Case/control | UK | 2363 | 4045 | 51[b] | 31 | T2D | ND | Q | 1.02 | | | | | NS |
| | Case/control | UK | 2363 | 4045 | 51[b] | 31 | T2D | ND | Q risk HT | 1.10 | | | | | NS |
| | Family based | UK | | | | | | | | | 529 | 41 | Q | T2D | NS |
| | Family based | UK | | | | | | | | | 529 | 41 | Q risk HT | T2D | NS |
| | Meta analysis [128-130,133,136,138-141] | Italy, Finland, Sweden, Denmark, Dominican Republic, South Asia, US, France, Austria, UK | ? | ? | ? | ? | T2D | ND | Q | 1.25 | | | | | 0.0007 |
| | Case/control | UK | 1500 | 1115 | ? | ? | Moderate obese (BMI 30-40) | Non-obese (BMI<25) | Q | 1.00 | | | | | NS |
| | Case/control | UK | 1500 | 1115 | ? | ? | Moderate obese (BMI 30-40) | Non-obese (BMI<25) | Q risk HT | 0.97 | | | | | NS |
| | Case/control | UK | 303 | 1115 | ? | ? | Morbid obese (BMI>40) | Non-obese (BMI<25) | Q | 0.92 | | | | | NS |
| | Case/control | UK | 303 | 1115 | ? | ? | Morbid obese (BMI>40) | Non-obese (BMI<25) | Q risk HT | 0.86 | | | | | NS |
| Keshavarz et al.[142] | Case/control | Japan | 348 | 369 | 63 | 36 | T2D | ND | QQ | 3.25 | | | | | 0.059 |
| | Case/control | Japan | 563 | 507 | 64 | 38 | T2D | ND | QQ | 0.45 | | | | | NS |
| | Case/control | All | 911 | 876 | 64 | 37 | T2D | ND | QQ | 1.14 | | | | | NS |
| | Quantitative | All | | | | | | | | | 1787 | ? | | BMI/ HbA1c | NS |

| Author | Study design | Population | # cases | # controls | Age cases | Age controls | Definition cases | Definition controls | Allele carriers | OR | # Quant | Mean age | Geno-type | Phenotype | *p* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Grarup et al.[143] | Case/control | Denmark | 1386 | 4770 | 52[b] | 46 | T2D | ND | Q | 1.02 | | | | | NS |
| | Case/control | Denmark | 570 | 2352 | ? | ? | T2D & BMI>30 | ND & BMI<25 | Q | ? | | | | | NS |
| | Meta analysis [128,129,131,135,136, 138-140,142,143] | Italy, Sweden, Canada, UK, Dominican Republic, US, South Asia, France, Austria, Japan, Denmark | 6466 | 11220 | ? | ? | T2D | ND | Q | 1.17 | | | | | $1*10^{-6}$ |
| | Case/control | Denmark | 3281 | 2582 | ? | ? | BMI≥25 | BMI<25 | QQ | 1.63 | | | | | 0.015 |
| | Case/control | Denmark | 969 | 2582 | ? | ? | BMI≥30 | BMI<25 | QQ | ? | | | | | NS |
| | Case/control | Denmark | 969 | 4894 | ? | ? | BMI≥30 | BMI<30 | QQ | ? | | | | | NS |
| | Quantitative | Denmark | | | | | | | | | 5863 | 46 | | Obesity/ glucose/ insulin parameters | NS |
| Matsuoka et al.[144] | Case/control | US (Caucasian) | 329 | 341 | 49 | 48 | Obese (mean BMI=39) | Non-obese (mean BMI=22) | Q | 0.70 | | | | | <0.05 |
| | Case/control | US (African American) | 169 | 152 | 44 | 46 | Obese (mean BMI=39) | Non-obese (mean BMI=24) | Q | 0.68 | | | | | <0.05 |
| | Quantitative | US (Caucasian & African American) | | | | | | | | | 991 | ±47 | XQ | Lower BMI | 0.02 |

| Author | Study design | Population | # cases | # controls | Age cases | Age controls | Definition cases | Definition controls | Allele carriers | OR | # Quant | Mean age | Geno-type | Phenotype | *p* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| ◆Prudente et al.[145] | Quantitative | Italy (east coast) | | | | | | | | | 631 | 37 | QQ | Lower BMI/waist/ glucose/ insulin/lipid parameters | 0.022/ 0.025 NS |
| | Quantitative | Italy (Sicily) | | | | | | | | | 304 | 38 | QQ | Lower BMI/ glucose/ insulin/lipid parameters | NS |
| | Quantitative | US (Dallas) | | | | | | | | | 505 | 46 | QQ | Lower BMI/ glucose/ insulin/lipid parameters | NS |
| | Quantitative | All | | | | | | | | | 1440 | 40 | QQ | Lower BMI/ glucose/ insulin/lipid parameters | 0.029 NS |
| Chandalia et al.[146] | Quantitative | US (Non-Hispanic whites) | | | | | | | | | 1038 | 45 | QQ | Higher BMI/waist/ glucose | <0.05 |
| | Quantitative | US (Hispanics) | | | | | | | | | 597 | 40 | QQ | Higher BMI/waist glucose/ insulin parameters | <0.05 NS |
| | Quantitative | US (African-Americans) | | | | | | | | | 1815 | 45 | | BMI/ glucose/ insulin parameters | NS |
| | Case/control | All | 358 | 3092 | | | T2D | ND | QQ | 1.?? | | | | | <0.05 |
| Gouni-Berthold et al.[147] | Case/control | Germany | 402 | 432 | 63 | 64 | T2D | ND | Q | 1.?? | | | | | NS |
| | Quantitative | Germany | | | | | | | | | 432 | 64 | | BMI | NS |
| | Quantitative | Germany | | | | | | | | | 402 (T2D) | 63 | | BMI | NS |

| Author | Study design | Population | # cases | # controls | Age cases | Age controls | Definition cases | Definition controls | Allele carriers | OR | # Quant | Mean age | Geno-type | Phenotype | *p* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Lyon et al.[148] | Case/control | US (Caucasians) & Poland | 1918 | 955 | 57 | 57 | Obese (mean BMI=35) | Non-obese (mean BMI=22) | Q or risk HT | 0.98<br>0.96 | | | | | NS<br>NS |
| | Case/control | African American | 96 | 92 | 38 | 41 | Obese (mean BMI=43) | Non-obese (mean BMI=21) | Q | 1.26 | | | | | NS |
| | Family based | African American | | | | | | | | | 846 | 38 | Q or risk HT | BMI | NS |
| | Case/control | US (Caucasians) | 1226 | 1226 | 63 | 61 | T2D | ND | Q or risk HT | 0.83<br>0.85 | | | | | 0.02/ NS |
| | Case/control | Poland | 1009 | 1009 | 62 | 59 | T2D | ND | Q or risk HT | 0.87<br>0.89 | | | | | NS |
| | Case/control, trios & sibships | Scandinavia/ Canada (n=4206) | ? | ? | ? | ? | T2D/ severe IGT | ND | Q or risk HT | 1.11<br>1.06 | | | | | NS |
| | Meta-analysis | All (n=8676) | ? | ? | ? | ? | T2D/ severe IGT | ND | Q or risk HT | 0.94<br>0.92 | | | | | NS |
| | Quantitative | Scandinavia | | | | | | | | | 1428 (T2D) | ?<br>? | | BMI<br>Glucose | NS<br>NS |
| | Quantitative | US (Caucasians) | | | | | | | | | 1226<br>1226 (T2D) | 61<br>63 | | BMI<br>Glucose | NS<br>NS |
| | Quantitative | Poland | | | | | | | | | 1009<br>1009 (T2D) | 59<br>62 | | BMI<br>Glucose | NS<br>NS |
| Bochenski et al.[149] | Case/control | Poland | 426 | 370 | 49[b] | 50 | T2D | ND | Q | ? | | | | | NS |
| | Case/control | Poland | 398 | 398 | 53 | 56 | Non-obese | Obese | Q | ? | | | | | NS |
| | Case/control | Poland | 191 | 207 | 51[b] | 52 | Non-obese & T2D | Non-obese & ND | Q | 0.58 | | | | | 0.05 |
| | Case/control | Poland | 235 | 163 | 48[b] | 47 | Obese & T2D | Obese & ND | Q | 1.70 | | | | | <0.05 |

| Author | Study design | Population | # cases | # controls | Age cases | Age controls | Definition cases | Definition controls | Allele carriers | OR | # Quant | Mean age | Geno-type | Phenotype | *p* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| ◆Chen et al.[150] | Case/control | China | 844 | 1862 | 62 | 65 | T2D | ND | Q | 0.96 | | | | | NS |
| | Meta-analysis [128-130,133,136,139,140,144] | Italy, Finland, Sweden, Denmark, Dominican Republic, US (Caucasian & African American), South Asia, China | 4766 | 6210 | ? | ? | T2D | ND | Q | 1.18 | | | | | 0.002 |
| | Quantitative | China | | | | | | | | | 1862 (T2D) | | | Obesity/glucose /insulin/lipid parameters | NS |
| | Quantitative | China | | | | | | | | | 844 | | | Obesity/glucose /insulin/lipid parameters | NS |
| | Quantitative | China | | | | | | | | | 2706 | | | Obesity/glucose /insulin/lipid parameters | NS |
| ◆Böttcher et al.[151] | Quantitative | Germany | | | | | | | | | 712 | 12 | | BMI | NS |
| | Case/control | Germany | 205 | 492 | 12 | 12 | Obese | Non-obese | Q or risk HT | 1.82<br>1.75 | | | | | 0.0005/<br>0.006 |
| | Case/control | Germany | 96 | 492 | 12 | 12 | Obese | Non-obese | Q or risk HT | 1.59<br>1.66 | | | | | 0.04/<br>0.066 |
| | Case/control | Germany | 195 | 492 | 11 | 12 | Obese | Non-obese | Q or risk HT | 1.49<br>1.47 | | | | | 0.03/<br>0.09 |
| | Quantitative | Germany | | | | | | | | | 205 (obese) | 12 | | Obesity/glucose /insulin/lipid parameters | NS |

| Author | Study design | Population | # cases | # controls | Age cases | Age controls | Definition cases | Definition controls | Allele carriers | OR | # Quant | Mean age | Geno-type | Phenotype | *p* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Quantitative | Germany | | | | | | | | | 205 (obese) | 12 | Q risk HT | Higher 2h glucose | <0.05 |
| ◆Meyre et al.[152] | Case/control | France | 1727 | 2005 | ? | ? | Overweight (BMI≥27) | Non-obese & ND | Q | 1.03 | | | | | NS |
| | Case/control | France | 784 | 2005 | ? | ? | Class I obesity (BMI≥30) | Non-obese & ND | Q | 0.99 | | | | | NS |
| | Case/control | France | 150 | 2005 | ? | ? | Class II obesity (BMI≥35) | Non-obese & ND | Q | 1.27 | | | | | NS |
| | Case/control | France | 986 | 2005 | ? | ? | Hypergly-caemic | Non-obese & ND | Q | 1.08 | | | | | NS |
| | Case/control | France | 316 | 2005 | ? | ? | T2D | Non-obese & ND | Q | 1.15 | | | | | NS |
| | Case/control | France | 265 | 2005 | ? | ? | Hypergly-caemic with T2D family history | Non-obese & ND | Q | 1.45 | | | | | 0.001 |
| | Case/control | France | 103 | 2005 | ? | ? | T2D with family history | Non-obese & ND | Q | 1.61 | | | | | 0.01 |
| | Quantitative | France | | | | | | | | | 4878 | 50 | Q | Higher BMI/ HDL:tria-cylglycerol ratio/stroke frequency | NS/ 0.02/ 0.03 |
| Willer et al.[105] | Case/control | Finland | 1155 | 971 | 54[b] | 65 | T2D | ND | XQ | 1.24 | | | | | 0.038 |
| ◆Leitão et al.[153] | Case/control | Brazil (European descendants) | 830 | 149 | 59 | ? | T2D | Anonymous blood donors | | | | | | | NS |
| | Case/control | Brazil (African descendants) | 197 | 91 | 55 | ? | T2D | Anonymous blood donors | | | | | | | NS |

| Author | Study design | Population | # cases | # controls | Age cases | Age controls | Definition cases | Definition controls | Allele carriers | OR | # Quant | Mean age | Geno-type | Phenotype | *p* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Quantitative | Brazil (European descendants) | | | | | | | | | 830 (T2D) | | | Obesity/ glucose/ insulin/lipid parameters | NS |
| | Quantitative | Brazil (African descendants) | | | | | | | | | 197 (T2D) | | | Obesity/ glucose/ insulin/lipid parameters | NS |
| ◆González-Sánchez et al.[154] | Case/control | Spain | 200 | 578 | 61 | 53 | Metabolic syndrome (MS) | Non-metabolic syndrome (NMS) | | | | | | | NS |
| | Quantitative | Spain | | | | | | | | | 809 (all) | 54 | | Obesity/ glucose/ insulin/lipid parameters | NS |
| | Quantitative | Spain | | | | | | | | | 200 (MS) | 61 | Q | Higher BMI /waist | 0.027/ 0.043 |
| | Quantitative | Spain | | | | | | | | | 578 (NMS) | 53 | | Obesity/ glucose/ insulin/lipid parameters | NS |
| | Quantitative | Spain | | | | | | | | | 73 (T2D) | 64 | Q | Higher BMI/leptin | 0.009/ 0.03 |
| | Quantitative | Spain | | | | | | | | | 721 (ND) | 53 | | Obesity/ glucose/ insulin/lipid parameters | NS |
| McAteer et al.[155] | Meta-analysis [105,128,129,133,135,138-141,143,146,148,149,152,155] | Europe (Italy, Scandinavia, UK, White American, France, Austria, Poland, | 12889 | 20363 | ? | ? | T2D | ND | QQ | 1.38 | | | | | 0.005 |

| Author | Study design | Population | # cases | # controls | Age cases | Age controls | Definition cases | Definition controls | Allele carriers | OR | # Quant | Mean age | Geno-type | Phenotype | *p* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Meta-analysis [105,128,129,133,135,138-141,143,146,148,149,152,155] | Denmark, Finland, Sweden) Europe (Italy, Scandinavia, UK, white American, France, Austria, Poland, Denmark, Finland, Sweden) | 12889 | 20363 | ? | ? | T2D | ND | QQ Adjusted for mean control BMI | 0.93 | | | | | 0.50 |
| Souren et al. (This study) | Quantitative (Twin study) | Belgium | | | | | | | | | ±600 | 25 | | Birth weight/obesity/ glucose/insulin/ lipid parameters | NS |
| | Quantitative (Twin study) | Belgium | | | | | | | | | 275 (men only) | 25 | Q | Lower NEFA | 0.02 |
| | Quantitative (Twin study) | Belgium | | | | | | | | | 315 (women only | 25 | Q | Higher HDL-cholesterol | 0.05 |

[a]Original association study. [b]Age at diagnosis. [c]After correction for multiple testing. AUC = area under the curve, HT = haplotype, ND = non-diabetic, NS = non-significant, OR = odds ratio, Quant = quantitative trait analysis, T2D = type 2 diabetes.

**Table S10.** Summary of published studies about the association between the *UCP2* (-866G>A) SNP and type 2 diabetes and/or related traits.

| Author | Study design | Population | # cases | # controls | Age cases | Age controls | Definition cases | Definition controls | Allele carriers | OR | # Quant | Mean age | Geno-type | Phenotype | *p* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| ◆Esterbauer et al.[156a] | Case/control | Austria, Germany | 340 | 256 | 41 | 40 | Obese (BMI>30) | Non-obese (BMI<30) | GA<br>AA | 0.61<br>0.60 | | | | | 0.007 |
| | Quantitative | Austria, Germany | | | | | | | | | 791 | 53 | A | Lower BMI Glucose intolerance/insulin resistance/lipid parameters | 0.03<br>NS |
| | Case-control | Austria, Germany | 109 | 589 | 54 | 53 | Obese (BMI>31) | Non-obese (BMI<29) | GA<br>AA | 0.58<br>0.66 | | | | | 0.06 |
| | Quantitative | | | | | | | | | | 96 | ? | A | Increased levels of intraperitoneal adipose tissue UCP2 mRNA | 0.02 |
| ◆Krempler et al.[157] | Quantitative | Austria, Germany | | | | | | | | | 39 | 35 | A | Lower disposition index<br>BMI/lipid parameters | 0.029<br>NS |
| | Case/control | Austria | 201 | 291 | 50[b] | 41 | Obese T2D | Obese | GA<br>AA | 2.10<br>2.12 | | | | | 0.003<br>0.035 |
| | Case/control | Austria | 201 | 100 | 50[b] | 54 | Obese T2D | Obese | GA<br>AA | 2.14<br>1.88 | | | | | 0.006<br>0.118 |
| Dalgaard et al.[158] | Case/control | Denmark | 749 | 816 | 43 | 48 | Obese men (BMI≥31) | Non-obese men | A | 0.95 | | | | | NS |
| | Quantitative | Denmark | | | | | | | | | 816 | 48 | AA | Higher BMI | 0.09 |
| | Quantitative | Denmark | | | | | | | | | 749 | 43 | AA | BMI | NS |
| | Quantitative | Denmark | | | | | | | | | 235 | 53 | | Obesity/glucose/ insulin parameters | NS |
| | Quantitative | Denmark | | | | | | | | | 410 | 60 | | Obesity/glucose/ insulin parameters | NS |

| Author | Study design | Population | # cases | # controls | Age cases | Age controls | Definition cases | Definition controls | Allele carriers | OR | # Quant | Mean age | Geno-type | Phenotype | *p* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| ◆Wang et al.[159] | Case/control | Utah (Caucasian) | 131 | 118 | 62 | 56 | T2D | ND | G | 1.43 | | | | | 0.05 |
| | Quantitative | Utah (Caucasian) | | | | | | | | | 661 (also T2D) | ±45 | | BMI/ triglycerides | NS |
| | Quantitative | Utah (Caucasian) | | | | | | | | | 355 | 43 | ? | Fasting glucose/insulin | 0.029/ NS |
| | Quantitative | Utah (Caucasian) | | | | | | | | | 125 | ? | GA | Lowest disposition index (heterozygosity effect) | 0.014 |
| | Quantitative | Utah (Caucasian, Asian, African American) | | | | | | | | | 36 | 38 | AA | Lower adipose UCP2 mRNA levels | 0.009 |
| ▲Reis et al.[160] | Quantitative | France | | | | | | | | | 385 (T2D) | 61 | A | Higher triglycerides/ total & LDL cholesterol | 0.008/ 0.039/ 0.08 |
| | | | | | | | | | | | 681 (T2D) | 61 | | BMI/fasting glucose | NS |
| ◆Mancini et al.[161] | Case/control | Italy | 198 | 374 | 40 | 45 | Moderate/ severe obese | Non-obese | A | 1.? | | | | | NS |
| | Quantitative | Italy | | | | | | | | | 374 | 45 | | Obesity/glucose/ lipid parameters | NS |
| | Quantitative | Italy | | | | | | | | | 122 (moderate obese) | 46 | | Obesity/glucose/ lipid parameters | NS |
| | Quantitative | Italy | | | | | | | | | 76 (severe obese) | 31 | | Obesity/glucose/ lipid parameters | NS |

| Author | Study design | Population | # cases | # controls | Age cases | Age controls | Definition cases | Definition controls | Allele carriers | OR | # Quant | Mean age | Geno-type | Phenotype | *p* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Sasahara et al.[162] | Case/control | Japan | 413 | 172 | 66 | 72 | T2D | ND | AA | 1.? | | | | | NS |
| | Quantitative | Japan | | | | | | | | | 413 (T2D) | 66 | XA | Earlier onset of diabetes/higher frequency of insulin therapy/BMI | 0.042/ 0.027/ NS |
| | Quantitative | Japan | | | | | | | | | 137 (T2D) | ±57 | XA | Lower glucose-induced insulin secretion | 0.009 |
| Le Fur et al.[163] | Case/control | Central Europe | 296 | 568 | 12 | ±30 | Obese | Never obese | | | | | | | NS |
| | Quantitative | Central Europe | | | | | | | | | 129 (Obese) | 12 | | BMI/glucose/ insulin parameters | NS |
| | Quantitative | Central Europe | | | | | | | | | 147 (Obese) | 12 | AA | Higher glucose oxidation rate/lower lipid oxidation rate | <0.05 |
| ◆Sesti et al.[164] | Quantitative | Italy | | | | | | | | | 302 | 42 | AA | Lower glucose-stimulated insulin release/lower disposition index | <0.05 |
| | Quantitative | Italy | | | | | | | | | 302 | 42 | AA | Obesity/lipid parameters | NS |
| Schauble et al.[165] | Case/control | Germany | 277 | 188 | ? | ? | Extremely obese | Never obese | | | | | | | NS |
| | Family based | Germany | | | | | | | | | 200 (obese) | ? | | | NS |
| Kovacs et al.[166] | Quantitative | Pima Indians | | | | | | | | | 263 | 26 | | Glucose/insulin parameters | NS |
| | Case/control | Pima Indians | 565 | 299 | ? | ? | T2D | ND | A | 1.05 | | | | | NS |
| | Quantitative | Pima Indians | | | | | | | | | 185 | 27 | G | Lower 24-h metabolic rate | 0.03 |

| Author | Study design | Population | # cases | # controls | Age cases | Age controls | Definition cases | Definition controls | Allele carriers | OR | # Quant | Mean age | Geno-type | Phenotype | *p* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| ◆D'adamo et al.[167] | Quantitative | Italy | | | | | | | | | 181 (ND offspring) | 37 | AA | Lower glucose disposal/insulin sensitivity index | 0.01/ 0.01 |
| | Case/control | Italy | 483 | 559 | 62 | 53 | T2D | ND | AA | 1.50 | | | | | 0.037 |
| | Case/control (women only) | Italy | 242 | 330 | 62 | 53 | T2D | ND | AA | 1.82 | | | | | 0.042 |
| | Quantitative | Italy | | | | | | | | | 565 | 53 | | BMI/glucose/lipid parameters | NS |
| | Quantitative | Italy | | | | | | | | | 483 (T2D) | 62 | | BMI/glucose/lipid parameters | NS |
| Ji et al.[168] | Case/control | Japan | 314 | 318 | 62 | ? | Hyper-tensive | Normo-tensive | A | 1.26 | | | | | <0.05 |
| | Case/control | Japan | 184 | 134 | 60 | 39 | T2D | ND + normo-tensive | A | 1.04 | | | | | NS |
| | Case/control | Japan | 158 | 134 | 60 | 39 | T2D + hyperten-sive | ND + normo-tensive | A | 1.28 | | | | | NS |
| | Quantitative | Japan | | | | | | | | | 184 (T2D only) | 60 | | BMI | NS |
| | Quantitative | Japan | | | | | | | | | 156 (hyperten-sive only) | 61 | | BMI | NS |
| | Quantitative | Japan | | | | | | | | | 158 (hyperten-sive and T2D) | 63 | | BMI | NS |
| | Quantitative | Japan | | | | | | | | | 134 (control) | 39 | | BMI | NS |
| | Quantitative | Japan | | | | | | | | | 111 (hyper-tensive without T2D | ? | | Insulin/HOMA-R | NS |

| Author | Study design | Population | # cases | # controls | Age cases | Age controls | Definition cases | Definition controls | Allele carriers | OR | # Quant | Mean age | Geno-type | Phenotype | *p* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| ◆Bulotta et al.[169] | Case/control | Italy | 746 | 327 | 60 | 45 | T2D | ND | GA<br>AA | 0.75<br>0.42 | | | | | 0.16<br>0.02 |
| | Quantitative | Italy | | | | | | | | | 746 (T2D) | 60 | | BMI/glucose/ insulin/lipid parameters | NS |
| | Quantitative | Italy | | | | | | | | | 327 | 45 | | BMI/glucose/ insulin/lipid parameters | NS |
| Gable et al.[170] | Case/control | UK | 169 | 2426 | 56 | 56 | Developed T2D (men) | ND | AA | 1.?? | | | | | NS |
| | Case/control | UK | 245 | 2426 | 56 | 56 | T2D (men) | ND | AA | 1.?? | | | | | 0.06 |
| Yoon et al.[171] | Quantitative | Korea (women) | | | | | | | | | 458 (overweight BMI>25) | 29 | A | Lower BMI/fat mass/protein mass | NS/ NS/ 0.03 |
| | Quantitative | Korea (women) | | | | | | | | | 301 (overweight subjects after weight control program) | 29 | A | Lower BMI change/fat mass change/protein mass change | 0.003/ 0.004/ NS |
| ◆Zurbano et al.[172] | Quantitative | Spain | | | | | | | | | 125 (obese) | 12 | A | Higher sum of tricipital and subscapular folds/ glucose/ insulin/lipid parameters | 0.034/ NS/NS/ NS |
| Ochoa et al.[173] | Case/control | Spain | 193 | 170 | 12 | 12 | Obese | Non-obese | GA | 0.?? | | | | | 0.038 |
| | Quantitative | Spain | | | | | | | | | 193 (obese) | 12 | | BMI/glucose/ insulin parameters | NS |
| | Quantitative | Spain | | | | | | | | | 170 | 12 | | | NS |

| Author | Study design | Population | # cases | # controls | Age cases | Age controls | Definition cases | Definition controls | Allele carriers | OR | # Quant | Mean age | Geno-type | Phenotype | *p* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Gable et al.[174] | Quantitative (North Europe) | UK, Sweden | | | | | | | | | 229 | ? | AA | Lower WHR | 0.03 |
| | Quantitative (North Europe) | UK, Sweden | | | | | | | | | 212 | ? | AA | Lower fasting insulin | 0.02 |
| | Quantitative (South Europe) | France, Italy | | | | | | | | | 197 | ? | AA | Lower WHR | 0.04 |
| | Quantitative (South Europe) | France, Italy | | | | | | | | | 202 | ? | AA | Lower fasting insulin | 0.37 |
| | Case/control | UK, Sweden, France, Italy | 598 | 635 | ? | ? | MI | Non-MI | AA | 1.?? | | | | | NS |
| ▲Shen et al.[175] | Quantiative | China | | | | | | | | | 1238 (men) | | AA | Higher waist/WHR | 0.016/ 0.018 |
| | Quantiative | Malay | | | | | | | | | 355 (men) | | | Obesity parameters | NS |
| | Quantiative | India | | | | | | | | | 265 (men) | | AA | Higher BMI/waist/ WHR/ triglycerides | 0.05/ 0.071/ 0.046/ 0.018 |
| | Quantitaive | All | | | | | | | | | 1858 (men) | | AA | Higher waist/WHR | 0.047/ 0.032/ |
| | Quantiative | China | | | | | | | | | 1498 (women) | | | Obesity/glucose/ lipid parameters | NS |
| | Quantiative | Malay | | | | | | | | | 381 (women) | | | Obesity/glucose/ lipid parameters | NS |
| | Quantiative | India | | | | | | | | | 281 (women | | | Obesity/glucose/ lipid parameters | NS |
| | Quantitaive | All | | | | | | | | | 2160 (women) | | | Obesity/glucose/ lipid parameters | NS |
| ▼Akrami et al.[176] | Quantitative | Iran | | | | | | | | | 75 | ? | GG | Higher HDL-cholesterol | 0.02 |

| Author | Study design | Population | # cases | # controls | Age cases | Age controls | Definition cases | Definition controls | Allele carriers | OR | # Quant | Mean age | Geno-type | Phenotype | *p* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Willer et al.[105] | Case/control | Finland | 765 | 602 | 51[b] | 68 | T2D | ND | XA | 1.21 | | | | | NS |
| ▲Cha et al.[177] | Quantitative | Korea | | | | | | | | | 658 (women) | 28 | AA | Lower HDL cholesterol | 0.003 |
| Vaxillaire et al.[101] | Case/control | France | 187 | 2919 | | | T2D | ND | | | | | | | NS |
| | Case/control | France | 336 | 2919 | | | IFG + T2D | ND | | | | | | | NS |
| | Quantitative | France | | | | | | | | | 2919 | ? | A | Higher BMI | 0.04 |
| | Quantitative | France | | | | | | | | | 2919 | ? | | Glucose/insulin parameters | NS |
| ▲Dhamrait et al.[178] | Case/control | UK | 204 | 2491 | 57 | 56 | CHD event | No CHD event | AA | 2.08 (HR) | | | | | <0.0001 |
| | Case/control | UK | 375 | 2316 | ±56 | ±56 | Obese | Non-obese | AA | 1.?? | | | | | 0.03 |
| ▲Oberkofler et al.[179] | Case/control | Austria | 404 | 461 | 51 | 47 | ACA (women) | No ACA | AA | 1.16 | | | | | 0.50 |
| | Case/control | Austria | 238 | 231 | 57 | 57 | ACA (male) | No ACA | AA | 2.01 | | | | | 0.01 |
| Souren et al. (This study) | Quantitative (Twin study) | Belgium | | | | | | | | | ±590 | 25 | | Birth weight/obesity/ glucose/insulin/ lipid parameters | NS |
| | Quantitative (Twin study) | Belgium | | | | | | | | | ±302 (women only) | 25 | XA | Higher LDL-cholesterol | 0.01 |

[a]Original association study. [b]Age at diagnosis. ACA = asymptomatic carotid atherosclerosis, CHD = coronary heart disease, IFG = impaired fasting glucose, MI = myocardial infarction, ND = non-diabetic, NS = non-significant, T2D = type 2 diabetes, OR = odds ratio, Quant = quantitative trait analysis.

**Table S11.** Summary of published studies about the association between the *FAAH* (P129T) SNP and type 2 diabetes and/or related traits.

| Author | Study design | Population | # cases | # controls | Age cases | Age controls | Definition cases | Definition controls | Allele carriers | OR | # Quant | Mean age | Geno-type | Phenotype | *p* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Sipe et al.[180a] | Case/control | US (White) | 1094 | 579 | ? | ? | Obese (BMI≥25) | Non-obese (BMI<25) | TT | 1.? | | | | | 0.005 |
| | Case/control | US (Black) | 507 | 104 | ? | ? | Obese (BMI≥25) | Non-obese (BMI<25) | TT | 1.? | | | | | 0.05 |
| | Case/control | Asian | 271 | 88 | ? | ? | (BMI≥23) | Non-obese (BMI<23) | TT | 0.? | | | | | NS |
| | Quantitative | All | | | | | | | | | 2667 | 57 | TT | Higher BMI | $<1*10^{-4}$ |
| Jensen et al.[181] | Case/control | Denmark | 3231 | 2507 | 47 | 45 | Overweight/ obese (BMI≥25) | Normal (BMI 18.5-<25) | T | 0.91 | | | | | 0.04 |
| | Case/control | Denmark | 959 | 2507 | 48 | 45 | Obese (BMI ≥30) | Normal (BMI 18.5<25) | T | 0.86 | | | | | 0.03 |
| | Case/control | Denmark | 2556 | 3180 | ? | ? | Waist circum-ference (≥80 in women and ≥94 in men) | Waist circumference (<80 in women and <94 in men) | T | 0.91 | | | | | 0.04 |
| | Quantitative | Denmark | | | | | | | | | 5801 | 46 | | Obesity, glucose/insulin/ lipid parameters | NS |
| Monteleone et al.[182] | Case/control | US (women) | 74 | 110 | 38 | 27 | Obese (mean BMI=43) | Non-obese (mean BMI=22) | T | 1.7 | | | | | 0.05 |
| | Case/control | US (women) | 189 | 110 | 36 | 27 | Obese women with or without binge eating disorder (mean BMI=40) | Non-obese (mean BMI=22) | T | 1.7 | | | | | 0.02 |

| Author | Study design | Population | # cases | # controls | Age cases | Age controls | Definition cases | Definition controls | Allele carriers | OR | # Quant | Mean age | Geno-type | Phenotype | *p* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Souren et al. (This study) | Quantitative (Twin study) | Belgium | | | | | | | | | ±590 | 25 | | Birth weight /obesity/ glucose/insulin/ lipid parameters | NS |
| | Quantitative (Twin study) | Belgium | | | | | | | | | 269 (men only) | 25 | T | Higher birth weight | 0.02 |

[a]Original association study. NS = non-significant, OR = odds ratio, Quant = quantitative trait analysis.

**Table S12.** Summary of published studies about the association between the *KCNJ11* (E23K) SNP and type 2 diabetes and/or related traits.

| Author | Study design | Population | # cases | # controls | Age cases | Age controls | Definition cases | Definition controls | Allele carriers | OR | # Quant | Mean age | Geno-type | Phenotype | *p* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Sakura et al.[183a] | Case/control | UK | 100 | 82 | 52[b] | ? | T2D | ND | | | | | | | NS |
| | Quantitative | UK | | | | | | | | | 100 (T2D) | 52 | | Obesity/glucose/ lipid parameters | NS |
| Hansen et al.[184] | Case/control | Denmark | 69 | 66 | ? | ? | T2D | ND | | | | | | | NS |
| | Quantitative | Denmark | | | | | | | | | 346 (ND) | 25 | | Obesity/glucose/ insulin parameters | NS |
| Inoue et al.[185] | Case/control | North-European | 306 | 175 | ? | ? | T2D | ND | | | | | | | NS |
| Hani et al.[186] | Case/control | France | 191 | 114 | 64 | 58 | T2D | ND | K | 1.?? | | | | | 0.015 |
| | Meta-analysis 183-185 | Denmark, UK, France | 521 | 367 | ? | ? | T2D | ND | K | 1.?? | | | | | 0.0016 |
| Gloyn et al.[187] | Case/control | UK | 360 | 307 | 53 | 55 | T2D | ND | KK | 2.?? | | | | | 0.0072 |
| | | | | | | | | | | | 360/ 307 | 53/ 55 | | Glucose intolerance/ insulin resistance parameters | NS |
| Love-Gregory et al.[188] | Case/control | Jewish | 272 | 402 | ? | ? | T2D | ND | K | 1.?? | | | | | NS |
| | Meta-analysis 183-185,187,188 | Denmark, UK, France, Jewish | 1153 | 1076 | ? | ? | T2D | ND | KK<br>K | 1.28<br>1.13 | | | | | $<1*10^{-4}$ |
| 't Hart et al.[189] | Case /control | The Netherlands | | | | | | | | | 65 (ND) | 46 | | Obesity/glucose /insulin parameters | NS |
| | | | | | | | | | | | 94 (IGT) | 57 | | Obesity/glucose /insulin parameters | NS |

| Author | Study design | Population | # cases | # controls | Age cases | Age controls | Definition cases | Definition controls | Allele carriers | OR | # Quant | Mean age | Geno-type | Phenotype | *p* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Nielsen et al.[190] | Quantitative | Denmark | | | | | | | | | 519 (ND) | 57 | EE | Lower BMI/ higher AUC for insulin/insulino-genic index | 0.013/ 0.014/ 0.022 |
| | Case/control | Denmark | 803 | 862 | 53[b] | 57 | T2D | ND | KK | 1.20 | | | | | NS |
| | Meta-analysis [185-187] | Denmark, UK, France, Utah | 1473 | 1351 | ? | ? | T2D | ND | KK | 1.49 | | | | | $2.2*10^{-4}$ |
| Gloyn et al.[191] | Case/control | UK | 622/ 232 | 855/ 327 | 56[b]/ 40[b] | 31/ ? | T2D/ T2D | ND/ ? | K | 1.18 | | | | | 0.01 |
| | TDT | UK | 150 | | 40 | | T2D | | | | | | | | NS |
| | All | UK | | | | | T2D | | K | 1.15 | | | | | 0.026 |
| | Quantitative | UK | | | | | | | | | 622/ 232/ 855/ 150 | 56/ 40/ 31/ ? | | BMI/WHR | NS |
| | Meta-analysis [183-187] | Denmark, UK, France, Utah | | | | | T2D | ND | K | 1.23 | | | | | $1.5*10^{-5}$ |
| Weedon et al.[192] | Quantitative | UK | | | | | | | | | 604 | 25 | | Birth weight/obesity/ glucose/insulin parameters | NS |
| | Quantitative | UK | | | | | | | | | 428 | 0 | | Birth weight | NS |
| Barroso et al.[135] | Case/control | UK | 517 | 517 | 56[b] | 64 | T2D | ND | KK | 1.49 | | | | | 0.0333 |
| Hansen et al.[193] | Case/control | Denmark | 1187 | 4791 | 50[b] | 46 | T2D | ND | K | 1.19 | | | | | 0.0002 |
| | Case/control | Denmark | 1187 | 1454 | 50[b] | 56 | T2D | ND | K | 1.15 | | | | | 0.02 |
| | | | | | | | | | | | 4273 | 45 | KK | Lower insulinogenic index | 0.007 |
| Yamada et al.[194] | Case/control | Japan | 193 | 73 | 63[b] | >60 | T2D | ND | | | | | | | NS |

| Author | Study design | Population | # cases | # controls | Age cases | Age controls | Definition cases | Definition controls | Allele carriers | OR | # Quant | Mean age | Geno-type | Phenotype | *p* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Florez et al.[195] | Discordant sib-pars | ? | 609 | 580 | 65 | 62 | T2D/ severe IGT | ND | | | | | | | |
| | Case/control | Scandinavia | 471 | 471 | 60 | 60 | T2D/ severe IGT | ND | | | | | | | |
| | Case/control | Canada | 127 | 127 | 53 | 52 | T2D | ND | | | | | | | |
| | Case/control | Sweden | 514 | 514 | 66 | 66 | T2D/ severe IGT | ND | | | | | | | |
| | All | | 1721 | 1692 | | | | | K | 1.17 | | | | | 0.003 |
| | Meta-analysis | | 5083 | 4747 | | | | | K | 1.15 | | | | | $<1*10^{-5}$ |
| | Quantitative (sib-pars) | | | | | | | | | | 44 (ND) | ? | K | Lower insulinogenic index | 0.006 |
| | Quantitative (sib-pars) | | | | | | | | | | 674 (ND) | ? | KK | Lower insulinogenic index | 0.008 |
| Le fur et al.[196] | Case/control | Central Europe | 388 | 178 | 12 | 12 | Obese | Non-obese | | | | | | | NS |
| | Quantitative | | | | | | | | | | 388 (obese) | 12 | | Obesity/ glucose/ insulin parameters | NS |
| Van Dam et al.[197] | Case/control | The Netherlands | 323 | 296 | 40-69 | 40-69 | T2D | ND | KK | 1.53 | | | | | NS |
| | Case/control | The Netherlands | 400 | 446 | 40-70 | 40-70 | IGT | ND | KK | 0.72 | | | | | NS |
| | Meta-analysis [135,183,185-187,190,191,197] | Denmark, UK, France Utah, The Netherlands | | | | | | | KK | 1.44 | | | | | 0.0007 |
| Xiong et al.[198] | Case/control | China | 119 | 101 | 59 | 57 | CHD | No CHD and ND | XK | 1.80 | | | | | 0.04 |
| | Quantitative | China | | | | | | | | | 119/ 101 | 59/ 57 | | Lipid parameters | NS |

| Author | Study design | Population | # cases | # controls | Age cases | Age controls | Definition cases | Definition controls | Allele carriers | OR | # Quant | Mean age | Geno-type | Phenotype | *p* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Ezenwaka et al.[199] | Case/control | Trinidad | 38 | 33 | 28 | 28 | Offspring of T2D patients | No family history | K | 1.?? | | | | | NS |
| | Quantitative | Trinidad | | | | | | | | | 70 (all) | 29 | EE | Lower HDL-cholesterol | <0.05 |
| | | | | | | | | | | | | | | Obesity/glucose /insulin/lipid parameters | NS |
| Cejkova et al.[200] | Case/control | Czech | 172 | 113 | 54[b] | ? | T2D | ND | | | | | | | NS |
| Yokoi et al.[201] | Case/control | Japan | 1590 | 1244 | 49[b] | ≥60 | T2D | ND | K | 1.08 | | | | | NS |
| Koo et al.[202] | Case/control | Korea | 758 | 630 | 59 | 65 | T2D | ND | K | 1.23 | | | | | 0.013 |
| | Quantitative | Korea | | | | | | | | | 630 (ND) | 65 | | Obesity/glucose /insulin/lipid parameters | NS |
| Scott et al.[55] | Case/control | Finland | 1161 | 1174 | 53[b] | 64 | T2D | ND | K | 1.20 | | | | | 0.0022 |
| | Case/control | Finland | 1215 | 1258 | 56[b] | 59 | T2D | ND | K | 1.04 | | | | | 0.55 |
| | Case/control | Finland | 2376 | 2432 | ±55 | ±62 | T2D | ND | K | 1.11 | | | | | 0.013 |
| | Meta-analysis | Finland, Sweden, US, Poland, UK | 10829 | 12622 | ? | ? | T2D | ND | K | 1.14 | | | | | $6.7*10^{-11}$ |
| Zeggini et al.[203] | Case/control | UK | 1924 | 2938 | 50[b] | ? | T2D | ? | K | 1.15 | | | | | $1.3*10^{-3}$ |
| | Meta-analysis | Finland, Sweden, US, Poland, UK | 10829 | 12622 | ? | ? | T2D | ND | K | 1.14 | | | | | $5.0*10^{-11}$ |
| Omori et al.[204] | Case/control | Japan | 1630 | 1064 | 62 | 46 | T2D | ND | K | 1.25 | | | | | 0.003 |
| | Quantitative | Japan | | | | | | | | | 1048 | 46 | KK | Lower BMI | 0.08 |

| Author | Study design | Population | # cases | # controls | Age cases | Age controls | Definition cases | Definition controls | Allele carriers | OR | # Quant | Mean age | Geno-type | Phenotype | *p* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Saxena et al.[56] | Case/control | Finland, Sweden | 1464 | 1467 | 58[b] | 58 | T2D | ND | K | 1.14 | | | | | 0.012 |
| | Case/control | Sweden, US, Poland | 5065 | 5785 | ±55 | ±60 | T2D | ND | K | 1.14 | | | | | $2.6*10^{-6}$ |
| | Case/control | Finland, Sweden, US, Poland | 6529 | 7252 | ±55 | ±60 | T2D | ND | K | 1.15 | | | | | $1*10^{-7}$ |
| | Meta-analysis | Finland, Sweden, US, Poland, UK | 10829 | 12622 | ? | ? | T2D | ND | K | 1.15 | | | | | $6.7*10^{-11}$ |
| Sakamoto et al.[205] | Case/control | Japan | 906 | 889 | 45[b] | 38 | T2D | ND | XK | 1.46 | | | | | 0.023 |
| | | | | | | | | | KK | 1.37 | | | | | 0.19 |
| | Quantitative | Japan | | | | | | | | | 906 (T2D) | | K | Higher glucose/ HbA1C | 0.004/ 0.001 |
| | Quantitative | Japan | | | | | | | | | 1795 (T2D+ ND ) | | K | BMI | NS |
| | Meta analysis [194,201,202] | East Asian | 3357 | 2836 | ? | ? | T2D | ND | XK | 1.23 | | | | | <0.0001 |
| Alsmadi et al.[206] | Case/control | Saudi Arabia | 550 | 335 | ≥60 | ≥60 | T2D | ND | K | 1.7 | | | | | 0.0001 |
| Vaxillaire et al.[101] | Case/control | France | 187 | 2919 | ? | ? | T2D | ND | K | 1.34 (HR) | | | | | 0.009 |
| | Case/control | France | 336 | 2919 | ? | ? | IFG + T2D | ND | K | 1.06 (HR) | | | | | NS |
| | Quantitative | France | | | | | | | | | 2919 | ? | | BMI/glucose/ insulin parameters | NS |

| Author | Study design | Population | # cases | # controls | Age cases | Age controls | Definition cases | Definition controls | Allele carriers | OR | # Quant | Mean age | Geno-type | Phenotype | *p* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Fischer et al.[207] | Case/control | Germany | 682 | 2263 | 55 | 50 | T2D | Subcohort of EPIC-Potsdam cohort (included 68 incident T2D cases) | KK | 1.25 | | | | | NS |
| | Case/control | Germany | 324 | 1070 | 60 | 51 | T2D | ND | KK | 1.85 | | | | | 0.007 |
| | Quantitative | Germany | | | | | | | | | 1567 (T2D and ND) | 57 | XK | Lower disposition index | 0.003 |
| Willer et al.[105] | Case/control | Finland | 1114 | 953 | 54[b] | 65 | T2D | ND | K | 1.22 | | | | | 0.0019 |
| Tschritter et al.[208] | Quantitative | German | | | | | | | | | 298 | 38 | XK | Higher glucose AUC during OGTT/lower insulin sensitivity/ greater glucagon AUC | 0.04 /0.05 /0.027 |
| | Quantitative | German | | | | | | | | | 75 | 39 | XK | Higher glucose AUC during hyperglycaemic / reduced glucagon decrease after glucose challenge | 0.02/ 0.06 |
| Souren et al. (This study) | Quantitative (Twin study) | Belgium | | | | | | | | | ±585 | 25 | | Birth weight /obesity/ glucose/ insulin/lipid parameters | NS |

[a]Original association study. [b]Age at diagnosis. CHD = coronary heart disease, IGT = impaired glucose tolerance, ND = non-diabetic, NS = non-significant, OGTT = oral glucose tolerance test, OR = odds ratio, Quant = quantitative trait analysis, T2D = type 2 diabetes.

**Table S13.** Summary of published studies about the association between the *AGTR1* (A1166C) SNP and type 2 diabetes and/or related traits.

| Author | Study design | Population | # cases | # controls | Age cases | Age controls | Definition cases | Definition controls | Allele carriers | OR | # Quant | Mean age | Geno-type | Phenotype | *p* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Abdollahi et al.[209a] | Quantitative | UK | | | | | | | | | 378 | 64.4 | CC | Lower BMI/ WHR/waist circumference/ body weight/ 30 min glucose/ fibrinogen | 0.01/ 0.004/ 0.001/ 0.008/ 0.01/ 0.03 |
| | | | | | | | | | | | 240 men only | ? | CC | Lower BMI/WHR/ waist circumference/ 30 min glucose/0 min insulin | 0.03/ 0.01/ 0.008 /0.01/ 0.04 |
| | | | | | | | | | | | 138 women only | ? | CC | Lower triacylglycerol/ fibrinogen | 0.04/ 0.01 |
| Souren et al. (This study) | Quantitative (Twin study) | Belgium | | | | | | | | | ±600 | 25 | | Birth weight /obesity/ glucose/ insulin/lipid parameters | NS |

[a]Original association study. Before this study, the *AGTR1* (A1166C) has frequently been studied in relation with hypertension. OR = odds ratio, Quant = quantitative trait analysis.

**Table S14.** Summary of published studies about the association between the *CYP1A1* (6235 T>C) SNP and type 2 diabetes and/or related traits.

| Author | Study design | Population | # cases | # controls | Age cases | Age controls | Definition cases | Definition controls | Allele carriers | OR | # Quant | Mean age | Geno-type | Phenotype | *p* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Chen et al.[210a] | Parent–offspring trios | China | 248 families | 248 families | | | Low birth weight <2500 gram | High birth weight ≥2500 gram | CC | 1.92 | | | | | 0.034 |
| Souren et al. (This study) | Quantitative (Twin study) | Belgium | | | | | | | | | | ±600 | 25 | Birth weight /obesity/ glucose/ insulin/lipid parameters | NS |

[a]Original association study. OR = odds ratio, Quant = quantitative trait analysis.

**Table S15.** Summary of published studies about the association between the *IRS2* (G1057D) SNP and type 2 diabetes and/or related traits.

| Author | Study design | Population | # cases | # controls | Age cases | Age controls | Definition cases | Definition controls | Allele carriers | OR | # Quant | Mean age | Geno-type | Phenotype | *p* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Bernal et al.[211a] | Case/control | Denmark | 252 | 267 | ? | ? | T2D | ND | | | | | | | NS |
| Stefan et al.[212] | Quantitative | Pima Indians | | | | | | | | | 233 | 28 | DD | Higher BMI/body fat/waist circum-ference | 0.02/ 0.01/ 0.004 |
| | Family based (n=998) | Pima Indians | ? | ? | ? | ? | Obese | Non-obese | | | | | | | NS |
| | Family based (n=998) | Pima Indians | ? | ? | ? | ? | T2D | ND | DD | 1.50 | | | | | 0.04 |
| Okazawa et al.[213] | Case/control | Japan | 123 | 260 | 60 | 51 | T2D | ND | DD | 1.13 | | | | | NS |
| | Case/control | Japan | 61 | 202 | ? | ? | T2D & BMI<25 | ND & BMI<25 | DD | 0.79 | | | | | NS |
| | Case/control | Japan | 62 | 58 | ? | ? | T2D & BMI≥25 | ND & BMI≥25 | DD | 1.89 | | | | | NS |
| | Quantitative | Japan | | | | | | | | | 260 | 51 | | Glucose/ insulin | NS |
| | Quantitative | Japan | | | | | | | | | 123 (T2D) | 60 | D | Glucose/ higher C-peptide | NS/ 0.011 |
| Mammarella et al.[214] | Case/control | Italy | 193 | 206 | 51[b] | 61 | T2D | ND | | | | | | | NS |
| | Case/control | Italy | 71 | 118 | 51[b] | 61 | T2D & BMI<27 | ND & BMI<27 | GD<br>DD | 0.46<br>0.18 | | | | | 0.0012 |
| | Case/control | Italy | 122 | 88 | 51[b] | 61 | T2D & BMI≥27 | ND & BMI≥27 | GD<br>DD | 2.50<br>5.74 | | | | | 0.0047 |
| | Quantitative | Italy | | | | | | | | | 102 | ? | GG | Higher C-peptide | 0.02 |
| | Quantitative | Italy | | | | | | | | | 116 (Obese T2D) | ? | DD | Higher glucose | 0.04 |

| Author | Study design | Population | # cases | # controls | Age cases | Age controls | Definition cases | Definition controls | Allele carriers | OR | # Quant | Mean age | Geno-type | Phenotype | *p* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Dálfonso et al.[215] | Case/control | Italy | 240 | 186+123 | 63 | 43 | T2D | ND | | | | | | | NS |
| | | | | | | | | | | | | | | | NS |
| | | | | | | | | | | | 240 (T2D)/ 186/ 123 | | | Obesity/ glucose/insulin/ lipid parameters | |
| Almind et al.[216] | Quantitative | Denmark | | | | | | | | | 363 | 25 | | BMI/insulin secretion | NS |
| | Quantitative | Denmark | | | | | | | | | 228 (ND offspring of T2D) | 39 | | and insulin sensitivity parameters | NS |
| | Quantitative | Denmark | | | | | | | | | 236 | 52 | DD | Lower BMI/ insulin/ C-peptide/30 min insulin/60 min insulin | 0.09/ 0.009/ 0.001/ 0.011/ 0.003 |
| | Quantitative | Sweden | | | | | | | | | 639 (men) | 71 | DD | Higher 60 min insulin/ BMI | 0.036/ NS |
| Fritsche et al.[217] | Quantitative | Germany | | | | | | | | | 318 | 34 | | Obesity/glucose/ insulin parameters | NS |
| | Quantitative | Germany | | | | | | | | | 77 | 38 | | Insulin secretion parameters of hyperglycaemic clamp | NS |
| Wang et al.[218] | Case/control | China | 100 | 85 | 60 | 52 | T2D | Healthy | | | | | | | NS |
| | Case/control | Finland | 85 | 82 | 67 | 54 | T2D | Healthy | | | | | | | NS |
| | Quantitative | | | | | | | | | | 333 | ±48 | | Glucose/insulin parameters | NS |
| Lautier et al.[219] | Case/control | France | 99 | 92 | 38 | 41 | Morbid obese (BMI>35) | Lean (mean BMI=23) | | | | | | | NS |

| Author | Study design | Population | # cases | # controls | Age cases | Age controls | Definition cases | Definition controls | Allele carriers | OR | # Quant | Mean age | Geno-type | Phenotype | *p* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 't Hart et al.[220] | Quantitative | The Netherlands | | | | | | | | | 64 | 46 | | Obesity/ glucose/insulin/ lipid parameters | NS |
| | Quantitative | The Netherlands | | | | | | | | | 94 (IGT) | 57 | | Obesity/glucose /insulin/lipid parameters | NS |
| Bodhini et al.[221] | Case/control | Asian Indians | 1018 | 1193 | 52 | 46 | T2D | ND | XG | 0.70 | | | | | 0.02 |
| | Case/control | Asian Indians | 532 | 783 | ? | ? | T2D (BMI<25) | ND (BMI<25) | DD | 0.82 | | | | | NS |
| | Case/control | Asian Indians | 486 | 410 | ? | ? | T2D (BMI≥25) | ND (BMI≥25) | DD | 2.19 | | | | | 0.002 |
| | Quantitative | Asian Indians | | | | | | | | | 1018 (T2D) | 52 | | Obesity/glucose /insulin/lipid parameters | NS |
| | Quantitative | Asian Indians | | | | | | | | | 1193 | 46 | | Obesity/glucose /insulin/lipid parameters | NS |
| Willer et al.[105] | Case/control | Finland | 1158 | 976 | 55[b] | 65 | T2D | ND | GG | 1.20 | | | | | 0.041 |
| Souren et al. (This study) | Quantitative (Twin study) | Belgium | | | | | | | | | ±585 | 25 | | Birth weight /obesity/ glucose/ insulin/lipid parameters | NS |

[a]Original association study. [b]Age at diagnosis. IGT = impaired glucose tolerance, ND = non-diabetic, NS = non-significant, OR = odds ratio, Quant = quantitative trait analysis, T2D = type 2 diabetes.

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# CHAPTER 5

# COMMON SNPS IN LEP AND LEPR ASSOCIATED WITH BIRTH WEIGHT AND TYPE 2 DIABETES RELATED METABOLIC RISK FACTORS IN TWINS

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## 5.1 ABSTRACT

Children born small for gestational age are at increased risk of developing T2D in adulthood. The satiety signal leptin that regulates food intake and energy expenditure might be a possible molecular link, since umbilical cord leptin levels are positively correlated with birth weight. In the present study we examined whether common single nucleotide polymorphisms (SNPs) in the leptin (*LEP*; 19G>A) gene and its receptor (*LEPR*; Q223R and K109R) are associated with birth weight and adult metabolic risk factors for T2D in twins. SNPs were genotyped in 396 monozygotic and 232 dizygotic twins (286 men and 342 women, mean age 25 years) recruited from the East Flanders Prospective Twin Survey. Data were analysed using linear mixed models. The *LEPR* K109R SNP was associated with birth weight (KK, KR and RR (95% CI): 2511 (2465-2557), 2575 (2516-2635) and 2726 (2606-2845) gram; $p_{additive}$=0.001). Also the *LEPR* Q223R SNP showed a significant association with weight at birth (QQ, QR and RR (95% CI): 2492 (2431-2554), 2545 (2495-2595) and 2655 (2571-2740) gram; $p_{additive}$=0.003). Furthermore, an interaction between the *LEPR* K109R and the Q223R SNP on birth weight was observed ($p$=0.014). G allele carriers of the *LEP* 19G>A SNP had higher HDL-cholesterol levels compared to 19A homozygotes (GX *vs* AA (95% CI): 1.62 (1.58-1.66) *vs* 1.49 (1.40-1.58) mmol/l; $p_{recessive}$=0.013). This study indicates that leptin may act as a growth-promoting signal during fetal development, and suggests a possible role for the *LEPR* in explaining the inverse relationship between birth weight and the development of metabolic diseases in adulthood. Additionally, these results suggest that the *LEP* 19G>A SNP affects HDL-cholesterol levels.

## 5.2 INTRODUCTION

Many studies have confirmed the association between low birth weight and increased risk of adult diseases like type 2 diabetes (T2D).[1-3] In an attempt to explain this association Hales and Barker[3] proposed the "thrifty phenotype hypothesis" and argued that insufficient nutrient supply during fetal life would lead to permanent changes of the structure and function of certain organs and tissues. These adaptations would result in an altered metabolic state, which is unfavourable in post-natal life when nutrient supply is abundant.[3] Genetic factors were not included in this hypothesis, even though there is substantial evidence that genetic susceptibility is important in the determination of both birth weight and adult metabolic disorders.[4,5] Hence, Hattersley and Tooke[6] suggested in the "fetal insulin hypothesis" that low birth weight and T2D have common genetic antecedents. This genetically determined insulin resistance may result in low insulin-mediated fetal growth as well as in insulin resistance in adulthood.[6] Although the hypothesis is supported by several single gene disorders,[6] the identification of variants that explain the association between low birth weight and T2D in the general population is less evident.

Molecular factors involved in fetal growth as well as in post-natal insulin metabolism are possible candidates for the "fetal insulin hypothesis". Leptin is a satiety factor that inhibits food intake and stimulates energy expenditure by acting on the appetite centres in the hypothalamic regions of the brain.[7] Leptin is primarily released by adipocytes and serum levels are highly correlated with adipose tissue mass. The hormone and its receptor are also expressed in other tissues, including pancreatic β cells where it inhibits insulin secretion.[7-9] During pregnancy leptin is produced by maternal and fetal adipose tissue as well as by the

placenta, and in contrast to maternal leptin levels, umbilical cord leptin levels are positively correlated with birth weight.[10,11] The association between single nucleotide polymorphisms (SNPs) in the leptin (*LEP*) or leptin receptor (*LEPR*) gene and birth weight has not been studied, this in contrast to the large number of (inconclusive) studies on their association with T2D and obesity.[12-39] Therefore, in this study we examined the effect of common SNPs in *LEP* and *LEPR* on birth weight and adult T2D related metabolic risk factors measured in young twins recruited from the East Flanders Prospective Twin Survey (EFPTS). The studied SNPs have previously been associated with T2D related traits in other populations,[19-37] and are either nonsynonymous or located in a region that might be of importance in the regulation of gene expression.

## 5.3 MATERIAL AND METHODS

### *5.3.1 Study population*

DNA was available of 628 individuals, including 197 monozygotic (MZ) (89 male and 108 female pairs) and 108 dizygotic (DZ) (33 male, 41 female and 34 mixed pairs) complete twin pairs, and 18 single twins (2 MZ/16 DZ) recruited from the population-based EFPTS. A detailed description of the twin sample has been given elsewhere.[4] Birth weights were obtained from obstetric records, and gestational age reported by the obstetrician was calculated as the number of completed weeks of pregnancy based on the last menstrual period. Maternal BMI (before pregnancy) and diabetes status was obtained from a questionnaire filled in by the mother of the twins, who was contacted at the time the twins were invited to the research centre. The methods used to measure the phenotypes at adult age have been described previously.[4] The Ethics Committee of the Faculty of Medicine of the Katholieke Universiteit Leuven approved the project and all participants gave informed consent.

### *5.3.2 DNA isolation*

Genomic DNA was initially extracted from available placental tissue collected at birth and/or twins were contacted and whole blood or mouth swabs were taken for DNA extraction. The DNA mini kit (Qiagen, Venlo, the Netherlands) was used for placental and mouth swab DNA extraction, the Wizard kit (Promega, Leiden, the Netherlands) was used for blood DNA extraction, both according to manufacturer's instructions. There is a possibility that placental tissues were switched within twin pairs during collection at birth. As a consequence genotypes and phenotypes would be combined incorrectly. To avoid false combinations, discordant genotypes within DZ pairs, of whom only DNA extracted from placenta tissue was available, were treated as missing values (less than 7% per SNP).

### *5.3.3 Genotyping*

The *LEP* 19G>A SNP located in the untranslated exon 1 (in *LEP* no common nonsynonymous SNPs have been identified), the *LEPR* K109R SNP in exon 4 and *LEPR* Q223R SNP in exon 6 were genotyped using pyrosequencing technology (Pyrosequencing AB, Uppsala, Sweden). PCRs were carried out in 15 µl mixes using on average 50 ng DNA and 5 pmol of forward

and reverse PCR primers (*LEP* 19G>A (rs2167270), GATCGGGCCGCTATAAGAG and CAGCTCCCGGTAACCTTCTA; *LEPR* K109R (rs1137100), CTTTTGCCTGCTGGACTCTC and GCTAATGCTTACCTATTTGTTGAAA; *LEPR* Q223R (rs1137101), AACAGCCAAACTCAACGACA and GCCACTCTTAATACCCCCAGT). The mix included 1x PCR buffer (Invitrogen, Breda, the Netherlands), 1.5 mM $MgCl_2$, 330 nM dNTPs (GE Healthcare, Eindhoven, the Netherlands) and 0.5 units of Taq polymerase (Invitrogen). DNA was denatured for 10 min at 95°C, followed by 50 cycles of 30 sec at 95°C, 30 sec at 55°C and 30 sec at 72°C, and a final extension step of 10 min at 72°C. Forward primers for the pyrosequencing assays were designed using Pyrosequencing Primer SNP Design Version 1.01 software (http://techsupport.pyrosequencing.com) (*LEP* 19G>A, AATCGCAGCGCCA; *LEPR* K109R, CAGACAACATTGAAGGAA; *LEPR* Q223R, CATCTGGTGGAGTAATTTT). Pyrosequencing was performed for sequence determination and allele designation in a Biotage PSQ HS 96A System according to the manufacturer's instructions. Prior to genotyping, 20 samples (including all 3 possible genotypes) were sequenced directly to validate the assays. No discrepancies were observed.

### 5.3.4 Statistical analysis

A $\chi^2$-test was used to check for Hardy-Weinberg proportions among genotype frequencies of the SNPs, using only one randomly selected twin per pair (Table S1). HAPLOVIEW was used to calculate linkage disequilibrium between the two SNPs in the *LEPR* gene.[40] Traits with a skewed distribution were normalised by log transformation. Differences in means between men and women according to zygosity were calculated using the PROC MIXED method implemented in the SAS package (version 9.1, SAS Institute Inc., Cary, NC, USA). A random intercept model was used, where the intercept of each twin pair was modelled as a function of the population intercept plus a unique contribution of the pair. Additionally, the variance-covariance structure of the random intercept was allowed to differ between MZ and DZ pairs.[41] For birth weight the intra-pair correlations differ between MZ monochorionic (MC) and MZ dichorionic (DC) twins. Since data on chorionicity were available, the variance-covariance structure of the random intercept of birth weight was allowed to differ between MZ MC, MZ DC and DZ pairs.

Before the association analyses were performed, the effects of potential covariates on all phenotypes were also checked using PROC MIXED. The phenotypes BMI, WHR and S4SF were checked for the effect of zygosity, gender and age; body mass, fat mass and lean body mass were checked for the effect of zygosity, gender, age and height; the blood parameters were checked for the effect of zygosity, gender, age and fat (BMI, WHR or S4SF) and birth weight was checked for the effect of gender, chorionicity and gestational age. Covariates were incorporated into the model, when the F-test indicated $p<0.10$. Subsequently, the genotype was entered into the optimised model as a class variable and a general association test (no assumption for mode of inheritance) was performed using a 2 *df* F-test. If the general association test was significant, then the mode of inheritance was further investigated by testing an additive, dominant and a recessive model using a 1 *df* F-test. In case of positive associations, possible SNP-SNP interactions (including interactions between SNPs within the same gene) were tested by including the main effects and the interaction term in the MIXED model.

### 5.3.5 Multiple testing

Since multiple tests were performed, a principal component factor analysis (PCFA) was carried out on all phenotypes to assess the number of separate hypotheses that were actually tested. The PCFA was performed on residuals adjusted for significant covariates and the number of factors were selected based on a criterion of eigenvalue >1.0. To produce interpretable factors we used an orthogonal Varimax rotation, since oblique rotation (i.e. Oblimin) showed that the inter-factor correlations were low (<0.10). In addition, the factor loadings yielded by oblique rotation were very similar to those produced by the Varimax rotation. Variables sharing at least 50% of their variance with a factor, equivalent to a factor loading >0.70, were considered as measuring the same construct and were therefore entered into the factor.
In total six factors were extracted by PCFA (Table S2). Factor 1 contained lean body mass and four obesity parameters, body mass, BMI, fat mass and S4SF. Factor 2 showed high loadings for total cholesterol and LDL-cholesterol. Factor 3 contained IGFBP1 and fasting glucose levels, and factor 4 fasting insulin and leptin levels. Factor 5 showed high loadings for HDL-cholesterol only, and in factor 6 no traits had a factor loading >0.70 (Table S2).
Within each factor, the number of phenotypes ($n_v$) was summed and to determine the significance threshold for these phenotypes a Bonferroni correction was performed ($\alpha/n_v$, where $\alpha$=0.05). Consequently, the significance threshold applied in the genetic association analysis for body mass, BMI, fat mass, lean body mass and S4SF was 0.01, and for total cholesterol, LDL-cholesterol, IGFBP1, fasting glucose, fasting insulin and leptin levels 0.025. The remaining phenotypes preserved the significance threshold of 0.05 (Table S2).

## 5.4 RESULTS

Phenotypic characteristics of the twins are summarised by gender in Table 1. In both MZ and DZ twins, men were taller and heavier and had higher lean body mass, WHR and fasting glucose levels than in women ($p$<0.05). On the other hand, fat mass, S4SF, IGFBP1, fasting insulin, leptin, HDL-cholesterol and NEFA levels were lower in men than in women ($p$<0.05). In MZ twins only, total cholesterol and triglycerides levels were significantly lower in men, and in DZ twins only, men had a higher weight at birth and higher LDL-cholesterol levels.
The major allele frequencies of the *LEP* 19G>A, *LEPR* K109R and *LEPR* Q223R SNPs were 0.627, 0.760 and 0.586, respectively, and are similar to those reported in other Caucasian populations (Table S1).[20,39] All genotype frequencies were in accordance with Hardy-Weinberg proportions ($p$>0.20). The pair wise linkage disequilibrium statistics D' and $r^2$ for the two *LEPR* SNPs were 0.93 and 0.40, respectively.
The R allele of the *LEPR* K109R SNP showed a significant association with birth weight (mean KK, KR and RR (95% CI): 2511 (2465-2557), 2575 (2516-2635) and 2726 (2606-2845) gram; $p_G$=0.003) (Table 2). In addition, the R allele of the *LEPR* Q223R SNP was also associated with a higher weight at birth (mean QQ, QR and RR (95% CI): 2492 (2431-2554), 2545 (2495-2595) and 2655 (2571-2740) gram; $p_G$=0.009) (Table 2). For both the *LEPR* K109R and the *LEPR* Q223R SNP the model assuming an additive mode of inheritance was most significant ($p_A$=0.001 and $p_A$=0.003, respectively). Between the *LEPR* SNPs and the adult metabolic risk factors no significant associations were observed (Table S3 en S4).

**Table 1.** Phenotypic characteristics of MZ and DZ twins by gender.

| | MZ | | | DZ | | |
|---|---|---|---|---|---|---|
| Characteristic | Males | Females | *p* | Males | Females | *p* |
| n | 179 | 217 | | 107 | 125 | |
| Birth weight (g) | 2540 ± 443 | 2456 ± 488 | 0.11 | 2690 ± 446 | 2572 ± 474 | 0.002 |
| Gestational age (wks)[a] | 36.9 ± 2.2 | 36.8 ± 2.6 | 0.98 | 37.4 ± 2.2 | 37.5 ± 2.3 | 0.82 |
| Age (yrs)[a] | 25.0 ± 4.6 | 24.8 ± 4.7 | 0.68 | 25.5 ± 4.7 | 25.6 ± 4.6 | 0.88 |
| Body height (cm) | 178.0 ± 5.9 | 165.3 ± 6.5 | <0.0001 | 178.5 ± 6.7 | 166.5 ± 6.5 | <0.0001 |
| Body mass (kg) | 69.3 ± 9.1 | 60.2 ± 9.9 | <0.0001 | 70.1 ± 10.1 | 60.7 ±10.0 | <0.0001 |
| BMI (kg/m$^2$)[b] | 21.7 ± 1.1 | 21.8 ±1.2 | 0.78 | 21.9 ± 1.1 | 21.7 ± 1.1 | 0.84 |
| Fat mass (kg)[b] | 11.3 ± 1.5 | 16.7 ± 1.3 | <0.0001 | 12.2 ± 1.5 | 16.6 ± 1.3 | <0.0001 |
| Lean body mass (kg) | 57.0 ± 6.2 | 42.8 ± 5.2 | <0.0001 | 57.0 ± 6.5 | 43.5 ± 5.9 | <0.0001 |
| S4SF (mm)[b] | 34.9 ± 1.5 | 56.2 ± 1.4 | <0.0001 | 34.2 ± 1.5 | 52.2 ± 1.4 | <0.0001 |
| WHR (%) | 82.9 ± 5.3 | 73.2 ± 4.5 | <0.0001 | 82.6 ± 5.1 | 72.3 ± 4.2 | <0.0001 |
| IGFBP1 (ng/ml)[b] | 10.9 ± 1.8 | 16.3 ± 2.0 | <0.0001 | 12.2 ± 1.8 | 19.1 ± 2.3 | <0.0001 |
| Fasting insulin (pmol/l)[b] | 32.5 ± 1.5 | 37.8 ± 1.6 | 0.005 | 31.6 ± 1.6 | 40.6 ± 1.5 | <0.0001 |
| Fasting glucose (mmol/l) | 5.0 ± 0.4 | 4.6 ± 0.4 | <0.0001 | 4.8 ± 0.4 | 4.6 ± 0.4 | <0.0001 |
| Leptin (ng/ml)[b] | 1.6 ± 3.1 | 11.8 ± 2.1 | <0.0001 | 1.6 ± 2.8 | 11.4 ± 1.9 | <0.0001 |
| Total cholesterol (mmol/l) | 4.8 ± 1.0 | 5.1 ± 0.8 | 0.003 | 5.0 ± 1.1 | 5.2 ± 1.0 | 0.08 |
| LDL-cholesterol (mmol/l) | 3.0 ± 0.9 | 2.9 ± 0.8 | 0.43 | 3.1 ± 1.0 | 2.9 ± 0.8 | 0.03 |
| HDL-cholesterol (mmol/l) | 1.4 ± 0.3 | 1.8 ± 0.4 | <0.0001 | 1.4 ± 0.4 | 1.9 ± 0.4 | <0.0001 |
| Triglycerides (mmol/l)[b] | 0.8 ± 1.5 | 0.9 ± 1.5 | 0.04 | 0.9 ± 1.5 | 0.9 ± 1.5 | 0.81 |
| NEFA (mmol/l) | 0.5 ± 0.2 | 0.7 ± 0.2 | <0.0001 | 0.5 ± 0.2 | 0.7 ± 0.2 | <0.0001 |

Data are expressed as mean ± SD. BMI = body mass index, IGFBP1 = insulin-like growth factor protein 1, n = number of individuals, NEFA = non-esterified fatty acids, S4SF = sum of four skinfolds, WHR = waist-to-hip ratio. [a]*p*-value calculated using standard linear regression, because convergence criteria could not be met in a random intercept model. [b]Geometric mean ± SD.

Because the presence of gestational diabetes might bias our results, we repeated the analysis without subjects of whom the mother had glycosuria during pregnancy (n=35 for K109R, n=33 for Q223R). In addition, 69 subjects were excluded because data on maternal diabetes status was missing. Also after exclusion of these subjects the association with birth weight remained significant for both the *LEPR* K109R and the *LEPR* Q223R SNP ($p_A$=0.004 and $p_A$=0.01, respectively).

Since maternal BMI is an important predictor of birth weight,[42] we repeated the analysis with maternal BMI as an extra covariate in the model. This resulted in a smaller sample size, because maternal BMI was missing for a number of individuals (n=105 for K109R, n=107 for Q223R). However, the association between birth weight and the *LEPR* K109R and the *LEPR* Q223R SNP remained significant ($p_A$=0.007 and $p_A$=0.05, respectively).

A significant interaction between the *LEPR* K109R and the *LEPR* Q223R SNP was observed for birth weight ($p$=0.014) (Figure 1). Subjects with the K109R/R223R or the R109R/R223R genotype had a significantly higher birth weight than subjects with the *LEPR* K109K/Q223Q, K109K/Q223R, K109K/R223R, K109R/Q223Q and the K109R/Q223R genotype (0.0006<$p$<0.05). In addition, subjects with the K109R/Q223Q genotype had a significantly lower birth weight compared to subjects with the K109K/Q223Q, K109K/Q223R, K109R/Q223R and R109R/Q223R genotype (0.007<$p$<0.05) (Figure 1).

**Table 2.** Significant associations between the SNPs in the *LEPR* and the *LEP* gene and T2D related metabolic risk factors; adjusted for significant covariates.

| Gene (SNP) | Trait[a] | Genotype | | | $p_G$ | $p_A$ | $p_D$ | $p_R$ |
|---|---|---|---|---|---|---|---|---|
| *LEPR* (K109R) | | KK | KR | RR | | | | |
| | n ($n_{MZ}/n_{DZ}$)[b] | 354 (240/114) | 194 (116/78) | 46 (30/16) | | | | |
| | Birth weight[c] | 2511 (2465-2557) | 2575 (2516-2635) | 2726 (2606-2845) | 0.003 | **0.001** | 0.01 | 0.003 |
| *LEPR* (Q223R) | | QQ | QR | RR | | | | |
| | n ($n_{MZ}/n_{DZ}$)[b] | 189 (126/63) | 295 (185/110) | 98 (66/32) | | | | |
| | Birth weight[c] | 2492 (2431-2554) | 2545 (2495-2595) | 2655 (2571-2740) | 0.009 | **0.003** | 0.04 | 0.006 |
| *LEP* (19G>A) | | GG | GA | AA | | | | |
| | n ($n_{MZ}/n_{DZ}$)[b] | 216 (131/85) | 265 (181/84) | 82 (53/29) | | | | |
| | HDL-cholesterol[d] | 1.59 (1.53-1.65) | 1.64 (1.59-1.69) | 1.49 (1.40-1.58) | 0.02 | 0.35 | 0.64 | **0.01** |

Data are expressed as least squares mean (95% confidence interval (CI)). [a]The units of the phenotypic characteristics are presented in Table 1. [b]Total number of individuals differ from the number reported in Table 1 because of missing values for the genotype, dependent variable or the covariates. [c]Adjusted for gender, gestational age and chorion type. [d]Adjusted for gender and WHR. n = total number of individuals, $n_{MZ}$ = number of MZ individuals, $n_{DZ}$ = number of DZ individuals, $p_A$ = *p*-value additive model, $p_D$ = *p*-value dominant model, $p_G$ = *p*-value general association model, $p_R$ = *p*-value recessive model. The lowest *p*-value is shown in **boldface type and underlined**. See also table S3, S4 and S5 of the supplementary information, for the means (95% CI) per genotype group and mode of inheritance (additive, dominant and recessive) of the associations that were considered non-significant.

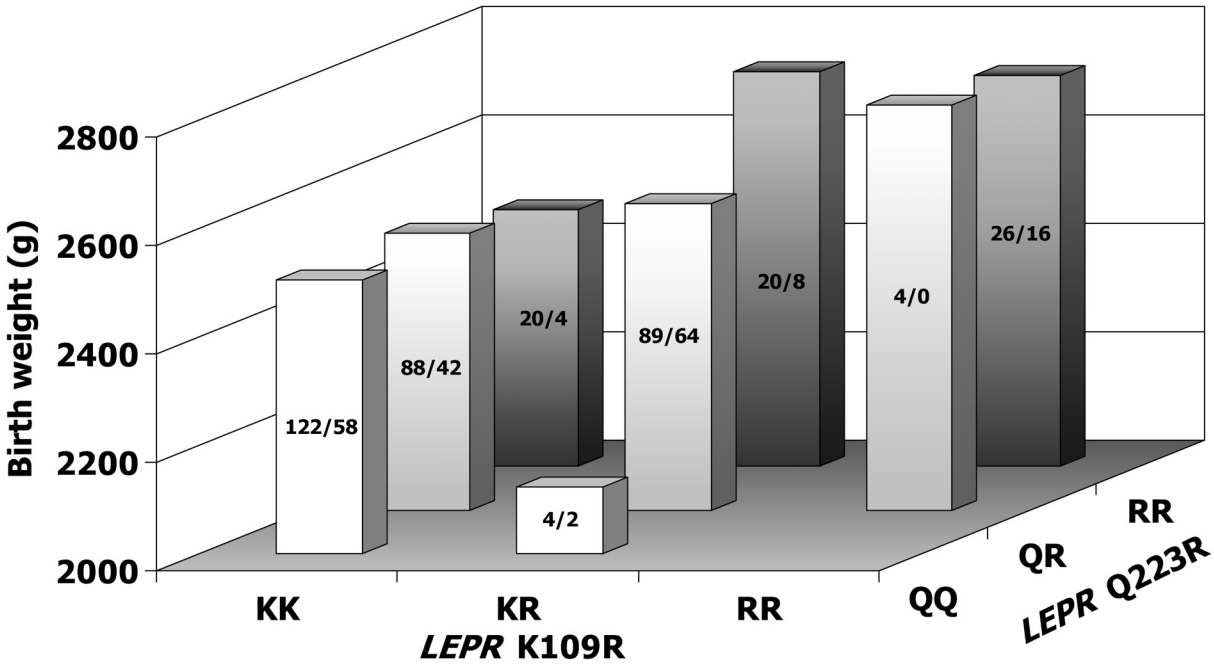


**Figure 1.** Interaction between the *LEPR* K109R and Q223R SNPs on birth weight adjusted for gender, gestational age and chorion type. Numbers in bars represent $n_{MZ}/n_{DZ}$, the number of MZ individuals / number of DZ individuals.

The *LEP* 19G>A SNP was significantly associated with HDL-cholesterol levels ($p_G$=0.02) (Table 2). Subjects carrying the G allele of *LEP* had higher HDL-cholesterol levels compared to 19A homozygotes (mean GX vs AA (95% CI): 1.62 (1.58-1.66) vs 1.49 (1.40-1.58) mmol/l; $p_R$=0.013). No significant associations between the *LEP* SNP and the other metabolic risk factors were observed (Table S5).

## 5.5 DISCUSSION

In this association study carried out in a young Belgian twin sample, we evaluated the effect of common SNPs in the *LEP* (19G>A) gene and its receptor (K109R and Q223R) on birth weight and T2D related metabolic risk factors measured in adult life.

The coding SNPs K109R and Q223R in the *LEPR* gene were strongly associated with birth weight; subjects carrying the R allele of the *LEPR* K109R and/or Q223R SNP had a significantly higher weight at birth. However, the two SNPs were moderately in linkage disequilibrium (D'=0.93 and $r^2$=0.40), suggesting that the observed associations are not completely independent. According to current literature, this is the first study investigating the relation between these *LEPR* SNPs and birth weight. Rand et al.[43] studied the relation between the Q223R SNP in the maternal *LEPR* gene and birth weight; fetal birth weight of mothers carrying the R allele tended to be higher in their UK cohort, but this relation was non-significant.[43] The functional relevance of the K109R and the Q223R SNP, both located within the extracellular domain of the leptin receptor, is largely unknown. However, the interaction observed in the present study between these two nonsynonymous SNPs on birth weight, may indicate that the binding affinity of the leptin receptor varies according to the different genotype groups.

Umbilical cord leptin levels are positively correlated with birth weight.[10,11] Currently, there is no consensus on the significance of this correlation, whether it implies that leptin acts as a growth-promoting signal during fetal development or whether it is just a good marker for fetal adiposity.[11] Our results, however, indicate that leptin does have a biologic function during prenatal growth. Interestingly, human subjects with total congenital leptin or a leptin receptor deficiency have a normal weight at birth, but exhibit rapid weight gain accompanied by an increased food intake in the first few months of life.[44,45] Since in postnatal life leptin regulates food intake by acting on the appetite centres in the hypothalamus, these rare cases indicate that leptin-mediated inhibitory effects on food intake are probably not functional in utero, which has also been demonstrated in near-term ovine foetuses.[46] Accordingly, it is more likely that the effect we observe is a result of peripheral leptin actions, for instance by modifying insulin secretion. During fetal life insulin is one of the major growth factors and it has been shown that leptin inhibits insulin release in pancreatic β cells.[6,8] A possible peripheral effect of leptin during fetal development is further supported by the observation that functional leptin receptors are already expressed in pancreatic islets of fetal rats.[47] Studying the relation between the *LEPR* SNPs and umbilical cord insulin levels might support the proposed mechanism.

The strong relation we observed between the *LEPR* SNPs and birth weight suggests that the *LEPR* is a good candidate gene for the "fetal insulin hypothesis". Under the assumption that the *LEPR* K109R and Q223R SNPs are indeed involved in the "fetal insulin hypothesis", according to our results, K allele carriers of the K109R SNP and Q allele carriers of the Q223R SNP are at increased risk for T2D and obesity. In the present study, K allele carriers of the K109R and Q allele carriers of the Q223R SNP tended to have lower leptin and higher NEFA levels, respectively (Table S3 and S4). These effects did however not reach the significance threshold defined for the general association test and were considered non–significant. The young age of the population and the moderate sample size, may have limited our statistical power to identify small effects. In literature, both alleles of these SNPs have been reported as risk alleles for T2D and obesity, and a meta-analytic study reported non-significant associations.[23-39,48] Consequently, additional studies in larger populations need to be carried out, to verify the involvement of these coding *LEPR* SNPs in the "fetal insulin hypothesis".

The G allele of the 19G>A SNP located in the 5' untranslated region of the *LEP* gene has been associated with both increased and reduced leptin levels and BMI, and a number of studies reported lack of association.[17-22] Since some studies used either small and/or selected samples and the criteria utilised to classify cases and controls differed largely, the relation between this SNP and T2D and/or obesity is still unclear. Our data suggest that the *LEP* 19G>A SNP may be protective against cardiovascular disease, as G allele carriers have significantly higher HDL-cholesterol levels. Although additional studies are required to confirm this relation, others have also reported a possible role for leptin in the HDL-cholesterol metabolism.[49] Greatly increased HDL-cholesterol levels have been observed in the severely obese leptin deficient *ob/ob* and the leptin receptor deficient *db/db* mice. This is in contrast with obese humans and other mouse models for obesity and diabetes that have low or normal HDL-cholesterol levels.[49] Functional studies have indicated that the high HDL-cholesterol levels in the *ob/ob* and *db/db* mice are probably due to a delayed hepatic HDL-cholesterol catabolism regulated by leptin.[49] It is striking that we observed association between the *LEP* 19G>A SNP and HDL-cholesterol levels, but not with leptin levels. Lack of

association with leptin levels might be due to low power, or might indicate that the *LEP* 19G>A SNP does not result in changes of *LEP* expression or translation in adipocytes, which are the main source of circulating leptin.
In conclusion, this study provides evidence that leptin acts as a growth-promoting signal during fetal development, and suggests a possible role for the *LEPR* gene in explaining the inverse relationship between birth weight and the development of adult metabolic disorders.

## 5.6 ACKNOWLEDGMENTS

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## 5.8 SUPPLEMENTARY MATERIAL

**Table S1.** Genotype frequencies and numbers, and allele frequencies of the SNPs calculated using a sub-sample that only included one randomly selected twin per pair.

| | | Homozygous major allele (n) | Heterozygous (n) | Homozygous minor allele (n) | Major allele | Minor allele |
|---|---|---|---|---|---|---|
| *LEP* | 19G>A | 0.387 (116) | 0.480 (144) | 0.133 (40) | 0.627 | 0.373 |
| *LEPR* | K109R | 0.590 (183) | 0.339 (105) | 0.071 (22) | 0.760 | 0.240 |
| *LEPR* | Q223R | 0.331 (100) | 0.510 (154) | 0.159 (48) | 0.586 | 0.414 |

**Table S2.** Factor loadings of the pre-diabetic phenotypes.

| | Factors | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | F | α |
| Birth weight | -0.03 | -0.05 | 0.14 | 0.13 | 0.19 | -0.67 | - | 0.05 |
| Body mass | **0.97** | 0.00 | -0.03 | 0.05 | -0.03 | -0.12 | 1 | 0.01 |
| BMI | **0.96** | -0.01 | -0.04 | 0.05 | -0.01 | -0.12 | 1 | 0.01 |
| Fat mass | **0.85** | 0.02 | 0.10 | 0.11 | 0.11 | 0.05 | 1 | 0.01 |
| Lean body mass | **0.80** | 0.00 | -0.13 | 0.03 | -0.09 | -0.26 | 1 | 0.01 |
| S4SF | **0.87** | -0.04 | 0.01 | -0.09 | 0.01 | 0.11 | 1 | 0.01 |
| WHR | 0.66 | 0.08 | 0.01 | 0.05 | -0.03 | 0.21 | - | 0.05 |
| IGFBP1 | -0.01 | -0.06 | **0.80** | -0.01 | 0.06 | 0.08 | 3 | 0.025 |
| Fasting glucose | 0.02 | -0.08 | **-0.74** | 0.16 | 0.08 | 0.08 | 3 | 0.025 |
| Fasting insulin | 0.07 | 0.02 | -0.23 | **0.78** | -0.13 | 0.17 | 4 | 0.025 |
| Leptin | 0.06 | -0.01 | 0.02 | **0.78** | 0.10 | -0.22 | 4 | 0.025 |
| Total cholesterol | 0.01 | **0.97** | 0.06 | 0.06 | 0.20 | 0.03 | 2 | 0.025 |
| LDL-cholesterol | 0.03 | **0.95** | -0.03 | -0.04 | -0.05 | -0.04 | 2 | 0.025 |
| HDL-cholesterol | -0.02 | 0.23 | 0.06 | 0.06 | **0.88** | 0.09 | 5 | 0.05 |
| Triglycerides | -0.02 | 0.33 | 0.36 | 0.33 | -0.57 | 0.23 | - | 0.05 |
| NEFA | -0.07 | -0.07 | 0.17 | 0.09 | 0.25 | 0.70 | - | 0.05 |
| Total variance (%) | 27.6 | 12.7 | 9.1 | 8.8 | 8.1 | 7.9 | - | - |
| Cumulative variance (%) | 27.6 | 40.3 | 49.4 | 58.2 | 66.3 | 74.2 | - | - |

Factor loadings >0.70 are shown in **boldface type**. α = significance threshold, F = factor.

**Table S3.** Association between T2D related metabolic risk factors and the *LEPR* K109R SNP.

| | | K109R | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Trait[a] | α | KK | KR | RR | $p_G$ | $p_A$ | $p_D$ | $p_R$ |
| n | | 358 | 198 | 46 | | | | |
| Birth weight | 0.05 | 2511 (2465-2557) | 2575 (2516-2635) | 2726 (2606-2845) | **0.003** | **0.001** | 0.01 | 0.003 |
| Body mass | 0.01 | 64.2 (63.1-65.3) | 65.3 (63.9-66.8) | 64.2 (61.3-67.2) | 0.44 | 0.48 | 0.28 | 0.74 |
| BMI[b] | 0.01 | 21.7 (21.3-22.1) | 22.0 (21.5-22.5) | 21.5 (20.6-22.5) | 0.50 | 0.78 | 0.47 | 0.51 |
| Fat mass[b] | 0.01 | 13.8 (13.2-14.3) | 14.2 (13.5-15.0) | 13.2 (11.8-14.7) | 0.35 | 0.90 | 0.48 | 0.33 |
| Lean body mass | 0.01 | 49.5 (48.8-50.1) | 50.0 (49.2-50.8) | 49.9 (48.3-51.5) | 0.52 | 0.32 | 0.26 | 0.81 |
| S4SF[b] | 0.01 | 43.4 (41.5-45.5) | 42.9 (40.4-45.5) | 39.5 (34.9-44.7) | 0.36 | 0.24 | 0.46 | 0.16 |
| WHR | 0.05 | 77.6 (77.0-78.1) | 77.8 (77.0-78.5) | 77.4 (75.9-78.9) | 0.87 | 0.88 | 0.73 | 0.78 |
| IGFBP1[b] | 0.025 | 14.1 (13.0-15.2) | 15.6 (14.1-17.2) | 13.2 (10.7-16.3) | 0.18 | 0.56 | 0.22 | 0.37 |
| Fasting glucose | 0.025 | 4.73 (4.68-4.78) | 4.71 (4.65-4.78) | 4.76 (4.62-4.89) | 0.86 | 0.94 | 0.88 | 0.64 |
| Fasting insulin[b] | 0.025 | 34.9 (33.3-36.7) | 36.7 (34.4-39.1) | 34.5 (30.2-39.5) | 0.43 | 0.55 | 0.31 | 0.66 |
| Leptin[b] | 0.025 | 4.15 (3.87-4.45) | 4.60 (4.20-5.04) | 5.06 (4.17-6.13) | 0.06 | 0.02 | 0.03 | 0.13 |
| Total cholesterol | 0.025 | 5.05 (4.93-5.16) | 4.95 (4.80-5.09) | 5.01 (4.71-5.31) | 0.52 | 0.40 | 0.28 | 0.98 |
| LDL-cholesterol | 0.025 | 3.00 (2.90-3.10) | 2.92 (2.79-3.05) | 2.90 (2.63-3.17) | 0.59 | 0.33 | 0.31 | 0.67 |
| HDL-cholesterol | 0.05 | 1.62 (1.57-1.66) | 1.60 (1.54-1.66) | 1.67 (1.54-1.81) | 0.59 | 0.75 | 0.92 | 0.34 |
| Triglycerides[b] | 0.05 | 0.86 (0.81-0.90) | 0.85 (0.80-0.91) | 0.97 (0.84-1.11) | 0.22 | 0.27 | 0.63 | 0.08 |
| NEFA | 0.05 | 0.61 (0.58-0.63) | 0.58 (0.54-0.61) | 0.59 (0.52-0.66) | 0.38 | 0.26 | 0.18 | 0.89 |

Data are expressed as least squares mean (95% CI). [a]The units of the phenotypic characteristics and abbreviation definitions are presented in Table 1. [b]Geometric least squares mean (95% CI). α=significance threshold, $p_A$ = *p*-value additive model, $p_D$ = *p*-value dominant model, $p_G$ = *p*-value general association model, $p_R$ = *p*-value recessive model. Associations are considered significant if the *p*-value of the general association test ($p_G$) is below the defined significance threshold (α).

**Table S4.** Association between T2D related metabolic risk factors and the *LEPR* Q223R SNP.

| | | Q223R | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Trait[a] | α | QQ | QR | RR | $p_G$ | $p_A$ | $p_D$ | $p_R$ |
| n | | 189 | 303 | 98 | | | | |
| Birth weight | 0.05 | 2492 (2431-2554) | 2545 (2495-2595) | 2655 (2571-2740) | **0.009** | **0.003** | 0.04 | 0.006 |
| Body mass | 0.01 | 64.5 (63.0-66.0) | 65.0 (63.8-66.3) | 64.7 (62.6-66.8) | 0.84 | 0.77 | 0.60 | 0.89 |
| BMI[b] | 0.01 | 21.8 (21.3-22.2) | 21.9 (21.6-22.3) | 21.7 (21.0-22.3) | 0.71 | 0.99 | 0.67 | 0.57 |
| Fat mass[b] | 0.01 | 13.8 (13.0-14.6) | 14.1 (13.5-14.8) | 13.9 (12.8-15.0) | 0.76 | 0.76 | 0.54 | 0.81 |
| Lean body mass | 0.01 | 49.8 (48.9-50.6) | 49.9 (49.2-50.6) | 49.6 (48.4-50.7) | 0.88 | 0.84 | 0.95 | 0.65 |
| S4SFb[b] | 0.01 | 43.6 (40.9-46.5) | 43.1 (41.0-45.4) | 42.6 (39.0-46.6) | 0.91 | 0.67 | 0.72 | 0.75 |
| WHR | 0.05 | 77.5 (76.7-78.3) | 77.7 (77.1-78.3) | 77.7 (76.6-78.8) | 0.90 | 0.71 | 0.65 | 0.93 |
| IGFBP1[b] | 0.025 | 13.4 (12.0-14.9) | 14.7 (13.6-16.0) | 14.0 (12.1-16.2) | 0.36 | 0.43 | 0.19 | 0.84 |
| Fasting glucose | 0.025 | 4.70 (4.63-4.77) | 4.74 (4.69-4.79) | 4.73 (4.63-4.82) | 0.62 | 0.50 | 0.34 | 0.98 |
| Fasting insulin[b] | 0.025 | 35.4 (33.1-37.9) | 35.4 (33.5-37.3) | 35.8 (32.6-39.4) | 0.97 | 0.89 | 0.99 | 0.82 |
| Leptin[b] | 0.025 | 4.32 (3.93-4.75) | 4.30 (3.99-4.63) | 4.79 (4.19-5.48) | 0.35 | 0.32 | 0.77 | 0.15 |
| Total cholesterol | 0.025 | 5.06 (4.91-5.21) | 4.95 (4.83-5.06) | 4.97 (4.76-5.18) | 0.48 | 0.36 | 0.23 | 0.90 |
| LDL-cholesterol | 0.025 | 2.98 (2.85-3.12) | 2.93 (2.82-3.04) | 2.92 (2.73-3.11) | 0.79 | 0.53 | 0.50 | 0.80 |
| HDL-cholesterol | 0.05 | 1.62 (1.56-1.69) | 1.60 (1.55-1.65) | 1.60 (1.50-1.69) | 0.81 | 0.56 | 0.52 | 0.79 |
| Triglycerides[b] | 0.05 | 0.88 (0.82-0.94) | 0.84 (0.79-0.88) | 0.91 (0.83-1.00) | 0.21 | 0.75 | 0.52 | 0.16 |
| NEFA | 0.05 | 0.62 (0.59-0.66) | 0.59 (0.56-0.62) | 0.57 (0.52-0.62) | 0.15 | 0.05 | 0.07 | 0.21 |

Data are expressed as least squares mean (95% CI). [a]The units of the phenotypic characteristics and abbreviation definitions are presented in Table 1. [b]Geometric least squares mean (95% CI). α=significance threshold, $p_A$ = *p*-value additive model, $p_D$ = *p*-value dominant model, $p_G$ = *p*-value general association model, $p_R$ = *p*-value recessive model. Associations are considered significant if the *p*-value of the general association test ($p_G$) is below the defined significance threshold (α).

**Table S5.** Association between T2D related metabolic risk factors and the 19G>A *LEP* SNP.

| | | 19G>A | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Trait[a] | α | GG | GA | AA | $p_G$ | $p_A$ | $p_D$ | $p_R$ |
| n | | 225 | 278 | 83 | | | | |
| Birth weight | 0.05 | 2554 (2497-2612) | 2548 (2496-2600) | 2501 (2409-2592) | 0.59 | 0.40 | 0.65 | 0.31 |
| Body mass | 0.01 | 65.1 (63.7-66.5) | 64.7 (63.4-65.9) | 64.1 (61.9-66.4) | 0.73 | 0.43 | 0.51 | 0.53 |
| BMI[b] | 0.01 | 22.0 (21.6-22.5) | 21.8 (21.3-22.2) | 21.7 (21.0-22.4) | 0.59 | 0.33 | 0.32 | 0.61 |
| Fat mass[b] | 0.01 | 14.2 (13.5-14.9) | 14.0 (13.3-14.7) | 13.5 (12.5-14.7) | 0.63 | 0.36 | 0.50 | 0.39 |
| Lean body mass | 0.01 | 50.2 (49.4-51.0) | 49.6 (48.9-50.3) | 49.2 (48.0-50.5) | 0.33 | 0.14 | 0.16 | 0.36 |
| S4SF[b] | 0.01 | 43.7 (41.2-46.3) | 43.6 (41.3-45.9) | 42.6 (38.9-46.7) | 0.90 | 0.70 | 0.84 | 0.65 |
| WHR | 0.05 | 77.8 (77.1-78.5) | 77.6 (76.9-78.2) | 77.8 (76.6-78.9) | 0.84 | 0.75 | 0.60 | 0.92 |
| IGFBP1[b] | 0.025 | 14.0 (12.8-15.4) | 14.9 (13.7-16.2) | 12.8 (11.0-14.9) | 0.22 | 0.63 | 0.70 | 0.14 |
| Fasting glucose | 0.025 | 4.76 (4.69-4.82) | 4.70 (4.64-4.76) | 4.74 (4.64-4.84) | 0.42 | 0.47 | 0.24 | 0.83 |
| Fasting insulin[b] | 0.025 | 35.1 (33.1-37.3) | 35.6 (33.7-37.6) | 38.2 (34.7-42.1) | 0.33 | 0.20 | 0.46 | 0.15 |
| Leptin[b] | 0.025 | 4.40 (4.03-4.80) | 4.33 (4.00-4.69) | 4.68 (4.07-5.39) | 0.63 | 0.61 | 0.96 | 0.36 |
| Total cholesterol | 0.025 | 5.08 (4.94-5.22) | 5.04 (4.91-5.17) | 4.79 (4.57-5.01) | 0.09 | 0.06 | 0.27 | 0.03 |
| LDL-cholesterol | 0.025 | 3.05 (2.92-3.17) | 2.95 (2.83-3.07) | 2.87 (2.67-3.07) | 0.29 | 0.12 | 0.16 | 0.28 |
| HDL-cholesterol | 0.05 | 1.59 (1.53-1.65) | 1.64 (1.59-1.69) | 1.49 (1.40-1.58) | **0.02** | 0.35 | 0.64 | **0.01** |
| Triglycerides[b] | 0.05 | 0.89 (0.84-0.95) | 0.87 (0.82-0.92) | 0.84 (0.76-0.93) | 0.57 | 0.29 | 0.37 | 0.42 |
| NEFA | 0.05 | 0.58 (0.55-0.62) | 0.60 (0.57-0.63) | 0.61 (0.55-0.66) | 0.72 | 0.42 | 0.43 | 0.64 |

Data are expressed as least squares mean (95% CI). [a]The units of the phenotypic characteristics and abbreviation definitions are presented in Table 1 of the manuscript. [b]Geometric least squares mean (95% CI). α=significance threshold, $p_A$ = *p*-value additive model, $p_D$ = *p*-value dominant model, $p_G$ = *p*-value general association model, $p_R$ = *p*-value recessive model. Associations are considered significant if the *p*-value of the general association test ($p_G$) is below the defined significance threshold (α).

# CHAPTER 6

# PARENT-OF-ORIGIN SPECIFIC LINKAGE AND ASSOCIATION OF THE *IGF2* GENE REGION WITH BIRTH WEIGHT AND ADULT METABOLIC RISK FACTORS

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*Submitted*

## 6.1 ABSTRACT

The maternally imprinted insulin-like growth factor 2 (*IGF2*) gene is an important fetal growth factor and is also suggested to have postnatal metabolic effects. In the present study we examined whether common genetic variants in *IGF2* (6815_6819delAGGGC, 1156T>C and 820G>A (*Apa*I)) and a microsatellite marker in the close vicinity of *IGF2* were linked to or associated with birth weight and adult metabolic risk factors. Genetic variants were genotyped in 199 monozygotic complete twin pairs, 109 dizygotic complete twin pairs, 15 single twins, 231 mothers and 228 fathers recruited from the East Flanders Prospective Twin Survey. Conventional and parent-of-origin specific linkage and association analyses were carried out with birth weight, adult body height and parameters quantifying obesity, insulin sensitivity and dyslipidaemia measured at adult age (mean age 25 years). In the parent-of-origin specific association analysis, in which only the paternally inherited allele was incorporated, the 1156T>C SNP showed significant association with IGF-binding protein 1 (IGFBP1) levels ($p$=0.005), and both the 6815_6819delAGGGC and 820G>A SNP showed a trend towards association with IGFBP1 levels ($p$=0.04 and $p$=0.09). In addition, the 6815_6819delAGGGC was significantly associated with total cholesterol ($p$=0.004) and LDL-cholesterol levels ($p$=0.007). No linkage was observed in either the conventional or in the parent-of-origin specific linkage analysis. This study suggests that paternally inherited alleles of common variants in the *IGF2* gene affect IGFBP1 and cholesterol levels.

## 6.2 INTRODUCTION

The insulin-like growth factor 2 (*IGF2*) gene is a complex transcription unit located in the imprinted 11p15.5 chromosomal region. *IGF2* is transcribed from five promoters (P0-P4) in a tissue- and development specific way resulting in mRNA transcripts with different 5' untranslated regions (UTRs) that encode the same IGF2 preprohormone (see Figure 1).[1,2] In most human fetal tissues *IGF2* is primarily transcribed from the P3 promoter and exclusively paternally expressed, with the exception of specific regions in the brain where both the maternal and paternal alleles are transcribed.[3] In human liver, the major production source of circulating IGF2, the *IGF2* gene is maternally imprinted during fetal life while postnatally *IGF2* is primarily transcribed from the P1 promoter, which is bi-allelicly active in the liver.[3,4] In other adult tissues, including heart, muscle, kidney and pancreas, *IGF2* is mainly transcribed from the paternally active promoters.[2,5]

Experiments in mice showed that IGF2 is an important fetal growth factor, since inactivation of the paternal *Igf2* allele results in significant growth retardation while over-expression of *Igf2* leads to fetal overgrowth.[6-8] In humans, low *IGF2* expression levels due to epigenetic modifications have been observed in patients with the Silver-Russel syndrome, which is characterised by severe intrauterine and postnatal growth retardation.[9,10] Conversely, over-expression of *IGF2* due to different reasons is frequently observed in patients with the Beckwith-Wiedermann syndrome, which is a fetal overgrowth disorder.[10,11]

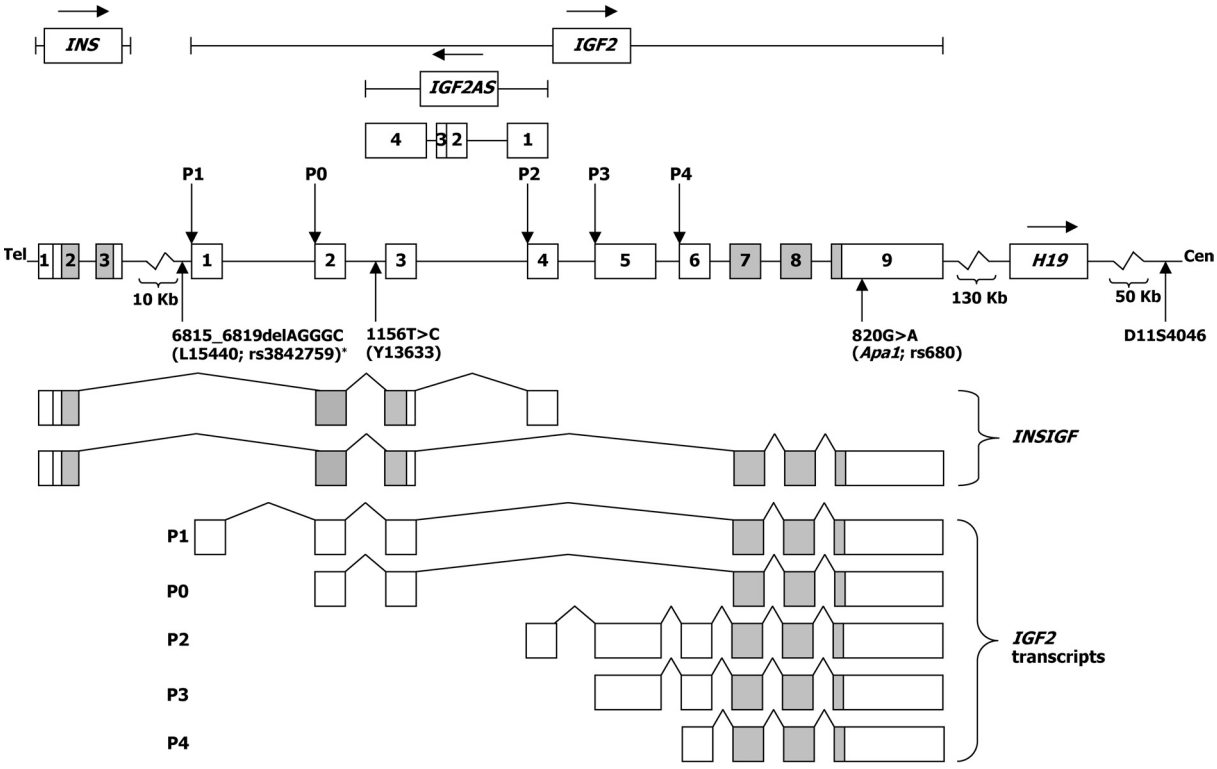


**Figure 1.** Schematic overview of the *IGF2* gene region and the variants genotyped in this study (adapted from Monk et al.[2] and Rodriquez et al.[12]). *IGF2* comprises 9 exons, 8 introns and 5 promoters (P0-P4). Precursor IGF2 is encoded by the shaded exons. IGF2AS = IGF2 antisense. *Sequencing showed that rs3842759 (6815A>T) is not a SNP but a 5 bp deletion (6815_6819delAGGGC).

Although *IGF2* is transcribed at a lower level after birth, several studies suggest that IGF2 may also have metabolic effects postnatally. For instance, transgenic mice over-expressing *Igf2* in the adult liver were found to have an increased insulin-stimulated glucose uptake and a lower fat body mass.[13,14] In addition, Kadlecová et al.[15] recently reported that a polymorphism in *Igf2* strongly segregates with hypertriglyceridaemia in rats. In pigs, a paternally expressed quantitative trait locus (QTL) affecting muscle growth, fat deposition and size of the heart, was explained by a substitution in intron 3 of the *IGF2* gene causing higher *IGF2* mRNA expression in postnatal cardiac and skeletal muscle, but not in liver.[16] Moreover, low levels of circulating IGF2 in human subjects with normal glucose tolerance and in lean type 2 diabetes patients have been associated with an increased risk of future weight gain.[17,18]

Since impaired fetal growth is a risk factor for adult metabolic diseases,[19] genes like *IGF2* are attractive candidates to explain susceptibility to disorders such as T2D and obesity, and accordingly several association studies have been performed.[20-30] In a sample of British middle-aged men the minor allele of the 820G>A (*Apa*I) single nucleotide polymorphism (SNP) located in the 3' UTR of the *IGF2* gene was associated with lower body mass, lower body mass index (BMI) and higher IGF2 levels.[21,22] Although the association with lower BMI was replicated in an Israeli sample,[23] several studies failed to confirm this.[24-26] Moreover, a study in American subjects even showed that minor allele carriers had a greater fat mass.[27]

In a follow up of the UK study, Gaunt et al.[28] systematically sequenced all (untranslated) exons and intron/exon boundaries of *IGF2* and showed that three (6815A>T and 1156T>C in the 5′ region, and 820G>A (*Apa*I)) out of eleven SNPs identified were independently associated with BMI. Recently, Heude et al.[29] failed to replicate these findings in 5000 middle-aged men and women, but did observe that these SNPs were significantly associated with body height. In addition, they reported that none of these SNPs were associated with birth weight, while in a Japanese sample minor allele carriers of the 820G>A (*Apa*I) SNP showed a significantly lower weight at birth.[30]
In summary, the studies published so far are inconclusive and none included imprinting effects. Furthermore, only 2 studies have examined the relation between *IGF2* SNPs and indices of the carbohydrate and lipid metabolism.[22,25] In the present study we genotyped the 6815A>T, 1156T>C, 820G>A (*Apa*I) SNPs and a microsatellite marker in the close vicinity of *IGF2* in young adult twins and their parents; and we carried out conventional and parent-of-origin specific linkage and association analyses with birth weight, adult body height and parameters quantifying obesity, insulin sensitivity and dyslipidaemia.

## 6.3 MATERIALS AND METHODS

### *6.3.1 Participants*

Twins and their parents were recruited from the EFPTS, which started in 1964 and has been recording all multiple births in the Belgian Province of East Flanders until the present.[31] For this study, DNA was available of 199 monozygotic (MZ) complete twin pairs, 109 dizygotic (DZ) complete twin pairs, 15 single twins (2 MZ/13 DZ), 231 mothers and 228 fathers. Of 70 complete twin pairs and 4 single twins no parental DNA was available, and of 37 complete twin pairs and 2 single twins DNA was only available of one parent. An overview of the families subdivided by available DNA samples is given in Table 1. Phenotypic information was only available from the twins (mean age 25 years). The methods used to measure the phenotypes are provided in the supplementary material. A more detailed description of the twin sample has been given in detail elsewhere.[31,32] The Ethics Committee of the Faculty of Medicine of the Katholieke Universiteit Leuven approved the project and the participants gave informed consent.

**Table 1.** Families categorised by available DNA samples.

| DNA available | Size | Number of families |
|---|---|---|
| Twins, father and mother | 4 | 201 |
| Twins and father only | 3 | 16 |
| Twins and mother only | 3 | 21 |
| Single twin, father and mother | 3 | 9 |
| Twins only | 2 | 70 |
| Single twin and father only | 2 | 2 |
| Single twin | 1 | 4 |
| Total | | 323 |

### 6.3.2 DNA extraction

For the twins, genomic DNA was initially extracted from available placental tissue collected at birth and/or twins were contacted and whole blood or mouth swabs were taken for DNA extraction. For the parents, mouth swabs were taken for DNA extraction. The DNA mini kit (Qiagen, Venlo, the Netherlands) was used for placental and mouth swab DNA extraction, the Wizard kit (Promega, Leiden, the Netherlands) was used for blood DNA extraction, both according to manufacturer's instructions. As there is a possibility that placental tissues were switched at birth within twin pairs, genotypes and phenotypes could possibly be combined incorrectly. To avoid false combinations, discordant genotypes within DZ pairs, of whom only DNA extracted from placenta tissue was available (43 individuals), were treated as missing values in the association analysis (less than 6% per SNP).

### 6.3.3 Genotyping

The 1156T>C SNP at nucleotide position 1156 in the GenBank sequence Y13633, the 6815A>T (rs3842759) and the 820G>A (*Apa*I) (rs680) SNP were selected for genotyping. These three SNPs have independently been associated with BMI in a large association study of Gaunt et al.[28] that systematically sequenced all (un)translated exons and intron/exon boundaries of *IGF2* (identified eleven SNPs in total). In addition, these three variants have been associated with body height in a large replication study by Heude et al.[29] Sequence analysis showed that the 6815A>T (rs3842759) variant in fact is a 5 bp deletion rather than a SNP. Therefore we have renamed this variant 6815_6819delAGGGC instead of 6815A>T (rs3842759). In order to estimate the proportion of alleles shared identical by descent (IBD) more properly, we genotyped the highly polymorphic (heterozygosity = 86%) microsatellite marker D11S4046, selected from the human UniSTS map (http://www.ncbi.nlm.nih.gov/genome/sts). The positions of the variants analysed in the *IGF2* gene region are shown in Figure 1.

For the twins, the 1156T>C and the 820G>A (*Apa*I) SNPs were genotyped using pyrosequencing technology (Pyrosequencing AB, Uppsala, Sweden). PCRs were carried out in 15 µl mixes using 50 ng genomic DNA and 5 pmol of forward and reverse PCR primers (Eurogentec, Seraing, Belgium), 1x PCR buffer and 0.5 U of *Taq* polymerase (Invitrogen, Breda, the Netherlands). Primer information and PCR conditions are listed in Table 2. Pyrosequencing primers were designed using Pyrosequencing Primer SNP Design Version 1.01 software (http://techsupport.pyrosequencing.com). Pyrosequencing was performed for sequence determination and allele designation in a Biotage PSQ HS 96A System according to the manufacturer's instructions.

For the parents, only very low amounts of DNA were available (derived from mouth swab extractions) and therefore the 1156T>C and the 820G>A SNPs were genotyped using the more sensitive SNaPshot assay. After amplification, the PCR products of both SNPs were pooled (2 µl of 820G>A and 3 µl of 1156T>C) and residual primers and unincorporated dNTPs were inactivated by incubating at 37°C for 3 h with 1.66 U of Antarctic Phosphatase and 0.66 U of Exonuclease I (New England Biolabs, Leusden, The Netherlands), followed by an inactivation step of 20 min at 80°C. The primers for single base extension (SBE) were designed to anneal to the anti-sense strand immediately adjacent to the SNP. To avoid overlap between the final extension products, the length of the extension primers was modified by adding a non-homologous tail (Table 2). SNaPshot reactions were carried out in

**Table 2.** Primer sequences and PCR conditions.

| Variant | Primer sequence (5'→3') | Size (bp) | $MgCl_2$ (mM) | dNTP[a] (mM) | # C[a] | T (°C) | Alleles |
|---|---|---|---|---|---|---|---|
| 6815_6819delAGGGC (rs3842759)[b] | F: FAM-TCAGCGGGTGACCCTAGC | 159-164 | 1.50[c] | 0.33 | 33 | 58 | 2 |
| | R: GAGGCCCAAGCTGGCAGT | | | | | | |
| 1156T>C | F: Biotin-GCCCAGATCCTGACAAGGT[d] | 164 | 1.50[c] | 0.33 | 50 | 55 | 2 |
| | R: ACACAAGCTCGGTGGTGAC | | | | | | |
| | PSQ: CCTCCCCATACACCC | | | | | | |
| | SBE: AAAAAAAAAAAAAAAAAAAAAAA-GGGACAGGGCTCAGGC | | | | | | |
| 820G>A (*Apa*I; rs680) | F: CTTGGACTTTGAGTCAAATTGG | 236 | 1.50[c] | 0.33 | 50 | 58 | 2 |
| | R: Biotin-CCTCCTTTGGTCTTACTGGG[d] | | | | | | |
| | PSQ: AGCAAAGAGAAAAGAAGG | | | | | | |
| | SBE: GACTGACT-CCAGCAAAGAGAAAAGAAGG | | | | | | |
| D11S4046 | F: FAM-ACTCCAGCCTGGGAAAC | 182-202 | 2.25 | 0.20 | 33 | 60 | 11 |
| | R: TGATAGACACACCCATTGC | | | | | | |

[a]DNA was denatured for 10 min at 95°C, followed by #C (cycles) of 30 sec at 95°C, 30 sec at T°C and 30 sec at 72°C, and a final extension step of 10 min at 72°C. [b]Sequencing showed that rs3842759 (6815A>T) is not a SNP but a 5 bp deletion (6815_6819delAGGGC). [c]10% DMSO. [d]For the SNaP-shot assay, primers without biotin label were used. C = cycles, F = forward, PSQ = pyrosequencing, R = reverse, SBE = single base extension, T = annealing temperature.

10 µl mixes containing 5 µl purified PCR product, 1 µl SNaPshot Ready Reaction Mix (Applied Biosystems, Nieuwerkerk a/d IJssel, the Netherlands), 1.6 µl pooled SBE primers and 2.4 µl water. Final SBE primer concentrations were 0.4 µM for 820G>A and 0.25 µM for 1156T>C. Thermocycling conditions were 1 cycle of 1 min at 96°C, 35 cycles of 10 sec at 96°C, 20 sec at 50°C and 30 sec at 60°C. Subsequently, extension products were incubated with 1 U of Shrimp Alkaline Phosphatase (ABgene, Surrey, UK) at 37°C for 1 hour, followed by 15 min at 75°C. Purified extension product were size-resolved by capillary electrophoresis on the ABI3100 Genetic Analyzer (Applied Biosystems). Size calling was performed with Genescan Software version 3.7 and the LIZ 120-size standard (Applied Biosystems).
For the 6815_6819delAGGGC and the D11S4046 microsatellite marker fluorescently-labeled primers were used to amplify the polymorphic DNA fragments (Table 2). The 5-times diluted FAM-labeled products were size-resolved by capillary electrophoresis on the ABI3100 Genetic Analyzer (Applied Biosystems). Fragment length was determined relative to the ROX 350-size standard using the Genescan Software version 3.7 (Applied Biosystems).

### 6.3.4 General statistical analysis

MERLIN was used to check for Mendelian errors.[33] A $\chi^2$-test was used to check Hardy-Weinberg equilibrium for the genotype frequencies of the genetic markers in the parent and the twin sample (using only one randomly selected co-twin per pair).

### 6.3.5 Conventional linkage analysis

To test for linkage between the *IGF2* region and the quantitative traits, multipoint variance components linkage analysis was performed using the statistical package MX.[34] In the models, phenotypic means were adjusted for significant covariates by modelling them as definition variables in the means model as previously described.[32] In the conventional variance components linkage analysis, phenotypic variation is decomposed into variance due to the QTL (Q), additive genetic (A, additive effects of genes on multiple loci), common environmental (C, environmental effects shared by twins reared in the same family) and unique environmental effects (E, environmental effects unique to the individual). Since MZ twins are genetically identical they are uninformative for linkage analysis. However, we included phenotypic data of MZ twins, as this will result in more accurate variance components estimates. For birth weight, the inclusion of phenotypic data of MZ monochorionic (MC) twins results in a reduced power to detect a QTL,[35] therefore only phenotypic data of the MZ dichorionic (DC) pairs were incorporated. Estimates of the variance component associated with the QTL were obtained using the $\hat{\pi}$-approach, in which the covariance due to the QTL for a sib-pair is modelled as a function of the proportion of alleles shared IBD.[36] The proportion of marker alleles shared IBD between twins is defined by $\hat{\pi}_k$, where $k$ refers to the $k$th twin pair. DZ twins may share 0, 1 and 2 alleles IBD, corresponding to $\hat{\pi}_k = 0$, 0.5 and 1.0, respectively. The effect of the QTL in the conventional analysis was evaluated by comparing a full model, in which the genetic variance caused by Q for a given phenotype was free, with a restricted model, in which the effect of Q was equal to zero. Logarithm of the odds (LOD) scores, computed as the difference in -2log likelihood divided by 4.6, were used to evaluate the QTL effect.

### 6.3.6 Parent-of-origin specific linkage analysis

To asses the parent-of-origin effect, the variance due to the QTL needs to be partitioned into a component that reflects the QTL effect of the maternally derived chromosome ($Q_M$) and a component reflecting the QTL effect of the paternally derived chromosome ($Q_P$). In addition, the proportion of marker alleles shared IBD $\hat{\pi}_k$ needs to be decomposed into a factor representing the proportion of maternal alleles shared IBD ($\pi_{Mk}$) and a factor representing the proportion of paternal alleles shared IBD ($\pi_{Pk}$). For DZ twins in a non-inbred family, four IBD states are possible: $\{i_M,j_M\}\{i_P,j_P\}$ (maternal and paternal alleles are shared IBD), $\{i_M,j_M\}\{i_P\}\{j_P\}$ (only maternal alleles are shared IBD), $\{i_M\}\{j_M\}\{i_P,j_P\}$ (only paternal alleles are shared IBD) and $\{i_M\}\{j_M\}\{i_P\}\{j_P\}$ (no alleles are shared IBD). Where *i* and *j* represent the members of a DZ twin pair, both carrying a maternally inherited allele ($i_M$ and $j_M$) and a paternally inherited allele ($i_P$ and $j_P$). The parent specific identity by descent $\pi_{Mk}$ is calculated by summing the probabilities of $\{i_M,j_M\}\{i_P,j_P\}$ and $\{i_M,j_M\}\{i_P\}\{j_P\}$; and $\pi_{Pk}$ is calculated by summing the probabilities of $\{i_M,j_M\}\{i_P,j_P\}$ and $\{i_M\}\{j_M\}\{i_P,j_P\}$. $\pi_{Mk}$ and $\pi_{Pk}$ can be either 0.0 and 0.5, because $\pi_{Mk} + \pi_{Pk} = \hat{\pi}_k$.

For the linkage analysis the D11S4046 marker is the most informative marker. Since the D11S4046 marker is also located close to the *H19* gene (Figure 1), which is maternally expressed only, we evaluated the effect of both $Q_P$ and $Q_M$. The effect of $Q_M$ (reported as $LOD_M$) was examined by comparing the full imprinting model with a model where $Q_M$ was equal to zero using the likelihood ratio $\chi^2$-test with 1 *df*.[37] $LOD_M$ was calculated as the difference in –2log likelihood divided by 4.6. The effect of $Q_P$ was computed in the same way and reported as $LOD_P$. In addition, the difference in fit between the non-nested full imprinting model and the conventional linkage model was evaluated using the Akaike's Information Criterion (AIC).[38]

Linkage analysis was performed using a multipoint IBD estimate based on the genotypes of the microsatellite marker and the three SNPs, which then represents the IBD state of the *IGF2* gene region. The probabilities of the different IBD states were estimated using MERLIN.[33] If parental genotypes were not available or not fully informative, then IBD states were estimated using allele frequencies computed using a sub-sample of one randomly selected co-twin per pair. Although linkage disequilibrium (LD) between the SNPs is low (max $r^2$=0.22), completely ignoring LD among the SNPs might overrate the IBD estimates, especially when parental data is missing. To overcome this problem, IBD estimates were calculated by taking marker-marker LD into consideration ($r^2$>0.10). Suggestive evidence for linkage was defined by LOD>1 and significant evidence for linkage by LOD>3.

### 6.3.7 Association analysis

Association between the *IGF2* SNPs and the quantitative traits was determined using the PROC MIXED method implemented in the SAS package (version 9.1, SAS Institute Inc., Cary, NC, USA). A random intercept model was used, where the intercept of each twin pair was modelled as a function of the population intercept plus a unique contribution of the pair. Additionally, the variance-covariance structure of the random intercept was allowed to differ between MZ and DZ pairs. For birth weight the intra-pair correlations differ between MZ MC and MZ DC twins. Since data on chorionicity were available, the variance-covariance structure of the random intercept of birth weight was allowed to differ between MZ MC, MZ DC and DZ pairs. The paternally inherited allele together with significant covariates (see

**Table 3.** Genotype and allele frequencies of the *IGF2* SNPs in the twins and their parents.

| | | Genotype | | | | Allele | | Paternal allele[a] | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | S | Homozygous major allele[b] ($n_M/n_W$) | Heterozygous[b] ($n_M/n_W$) | Homozygous minor allele[b] ($n_M/n_W$) | $p_{HWE}$ | Major Allele[b] | Minor allele[b] | Major allele (n) | Minor allele (n) | GR |
| 6815_6819delAGGGC | P | 0.527 (113/121) | 0.396 (92/84) | 0.077 (14/20) | 0.91 | 0.73 | 0.27 | | | 0.99 |
| | T | 0.578 (150/197) | 0.360 (109/109) | 0.062 (15/22) | 0.76 | 0.76 | 0.24 | 0.82 (420) | 0.18 (92) | 0.99 |
| 1156T>C | P | 0.417 (71/91) | 0.445 (89/84) | 0.139 (30/24) | 0.47 | 0.64 | 0.36 | | | 0.87 |
| | T | 0.418 (123/119) | 0.428 (94/147) | 0.154 (44/45) | 0.17 | 0.63 | 0.37 | 0.65 (311) | 0.35 (171) | 0.92 |
| 820G>A (*Apa*I) | P | 0.512 (105/104) | 0.380 (84/71) | 0.108 (13/31) | 0.06 | 0.70 | 0.30 | | | 0.91 |
| | T | 0.515 (143/170) | 0.412 (103/130) | 0.073 (25/20) | 0.65 | 0.72 | 0.28 | 0.79 (392) | 0.21 (103) | 0.98 |

[a]Since some families were uninformative, the paternal derived allele could not always be determined. [b]Frequencies for the twins are calculated using a sub-sample, that only included one randomly selected twin per pair. See Table S3 for the numbers per genotype group of the sub-sample. GR = genotyping rate, $n_M$ = number of men in the whole sample, $n_W$ = number of women in the whole sample, P = parents, $p_{HWE}$ = *p*-value of the Hardy-Weinberg equilibrium test, S = sample, T = twins.

Table S1) were incorporated into the random intercept model and association was tested using a 1 *df* F-test. In addition, genotypes based on both parental alleles were incorporated into the random intercept model and a general association test (no assumption for mode of inheritance) was performed using a 2 *df* F-test. An association was considered significant if the *p*-value was below the significance threshold defined that was adjusted for multiple testing, of which a detailed description is provided in the supplementary material.

## 6.4 RESULTS

Sequence analysis showed that the 6815A>T (rs3842759) variant in fact is a 5 bp deletion instead of a single nucleotide substitution, therefore we have renamed this variant 6815_6819delAGGGC. Genotype and allele frequencies of the polymorphisms in the twins and their parents are listed in Table 3. The allele frequencies of the D11S4046 microsatellite marker in the twins and their parents are presented in Table S4. All genotype frequencies were in agreement with Hardy-Weinberg equilibrium ($p$>0.05) and are similar to those reported in other Caucasian populations.[22,24-29] The 1156T>C SNP has thus far only been examined in two studies that reported conflicting genotype frequencies.[28,29] Our genotype

**Table 4.** Results of univariate multipoint variance components linkage analysis with and without incorporating the parent-of-origin effect: the fit of the models and the LOD scores.

| Model | A + E (AIC) | A + E + Q[a] (AIC) | A + E + $Q_P$ + $Q_M$[b] (AIC) | LOD | $LOD_P$ | $LOD_M$ |
|---|---|---|---|---|---|---|
| Birth weight | -550.34 | -548.36 | -546.58 | 0.01 | 0.00 | 0.05 |
| Body height | 2645.60 | 2647.60 | 2649.60 | 0.00 | 0.00 | 0.00 |
| Body mass | 3396.93 | 3398.93 | 3400.93 | 0.00 | 0.00 | 0.00 |
| BMI | -2455.37 | -2453.37 | -2451.37 | 0.00 | 0.00 | 0.00 |
| Fat mass | -1185.15 | -1183.15 | -1181.15 | 0.00 | 0.00 | 0.00 |
| Lean body mass | 2543.07 | 2544.62 | 2547.07 | 0.10 | 0.00 | 0.00 |
| S4SF | -1008.52 | -1006.52 | -1004.52 | 0.00 | 0.00 | 0.00 |
| WHR | 2515.68 | 2517.68 | 2519.61 | 0.00 | 0.00 | 0.02 |
| IGFBP1 | -62.80 | -60.89 | -61.48[c] | 0.02 | 0.37 | 0.01 |
| Fasting glucose | -804.21 | -802.21 | -800.26 | 0.00 | 0.01 | 0.00 |
| Fasting insulin | -712.49 | -710.49 | -708.67 | 0.00 | 0.00 | 0.04 |
| Leptin | -276.68 | -274.68 | -272.68 | 0.00 | 0.00 | 0.00 |
| Total cholesterol | 4994.63 | 4996.63 | 4998.63 | 0.00 | 0.00 | 0.00 |
| LDL-cholesterol | 4831.02 | 4832.82 | 4835.02 | 0.05 | 0.00 | 0.00 |
| HDL-cholesterol | 3876.43 | 3876.94 | 3878.69 | 0.33 | 0.37 | 0.00 |
| Triglycerides | -733.49 | -731.49 | -729.49 | 0.00 | 0.00 | 0.00 |
| NEFA | -1488.50 | -1486.50 | -1484.77 | 0.00 | 0.00 | 0.06 |

[a]Conventional linkage model. [b]Full imprinting model. [c]Better fit compared to the conventional linkage model. AIC = Akaike's information criterion (lower value indicates better model fit), BMI = body mass index, IGFBP1 = insulin-like growth factor protein 1, LOD = QTL effect, $LOD_M$ = QTL effect of the maternally derived chromosome, $LOD_P$ = QTL effect of the paternally derived chromosome, NEFA = non-esterified fatty acids, S4SF = sum of four skinfolds, WHR = waist-to-hip ratio.

frequencies were in agreement with those of Heude et al.[29] Also the frequency distributions of the paternally inherited alleles are listed in Table 3. Because some families were uninformative, the total number of individuals from whom the paternally inherited allele was known is lower than the number of individuals from whom a genotype was available.
The results of the univariate multipoint variance components linkage analysis with and without incorporating the parent-of-origin effect are presented in Table 4. No significant or suggestive linkages were observed in either the conventional linkage analysis or in the parent-of-origin specific linkage analysis.
The results of the parent-of-origin specific association analysis using the paternally inherited allele only are presented in Table 5. The 6815_6819delAGGGC was significantly associated with total cholesterol levels (wt and del (mean (95% CI)): 4.91 (4.80-5.01) and 5.24 (5.03-5.45) mmol/l, $p$=0.004) and LDL-cholesterol levels (wt and del: 2.89 (2.80-2.99) and 3.18 (2.99-3.37) mmol/l, $p$=0.007) (Table 5). The 1156T>C SNP was associated with IGF-binding protein 1 (IGFBP1) levels (T and C: 13.2 (12.2-14.4) and 16.0 (14.4-17.9) ng/ml, $p$=0.005). Interestingly, both the 6815_6819delAGGGC and the 820G>A SNP showed a trend towards association with IGFBP1 levels (wt and del: 14.5 (13.4-15.5) and 12.2 (10.5-14.2) ng/ml, $p$=0.04: G and A: 14.9 (13.8-16.0) and 13.0 (11.3-15.0) ng/ml, $p$=0.09) (Table 5).
The results of the general association analysis based on both parental alleles are presented in Table S5-S7. No significant associations were observed in this analysis.

## 6.5 DISCUSSION

In the present study we carried out conventional and parent-of-origin specific linkage and association analyses to study the relation between the 6815_6819delAGGGC, the 1156T>C SNP, the 820G>A (*Apa*I) SNP and the D11S4046 microsatellite marker, located in the *IGF2* gene region, with birth weight, adult body height and parameters quantifying obesity, insulin sensitivity and dyslipidaemia measured at adult age.
The 6815_6819delAGGGC, the 1156T>C and the 820G>A (*Apa*I) SNPs all have been independently associated with BMI in a large UK sample.[28] We could not replicate these associations in the present sample, nor the recently reported association by Heude et al.[29] with adult body height. As our study has a smaller sample size, less power might explain these negative findings. In addition, Kaku et al.[30] found that the minor allele of the 820G>A (*Apa*I) SNP was associated with a lower weight at birth in a Japanese sample.[30] Neither the present study nor the large study (n=5000) of Heude et al.[29] could replicate this association. These results might indicate that the association observed in the Japanese sample is a result of an ethnic-specific effect, i.e. a functional rare variant in linkage disequilibrium with the 820G>A (*Apa*I) SNP.
In the present study we did observe a very interesting association with IGFBP1 levels, since, carriers of the paternally inherited minor allele of the 1156T>C SNP had significantly higher IGFBP1 levels compared to carriers of the paternally inherited major allele ($p$=0.005). In addition, we observed for the 6815_6819delAGGGC and the 820G>A (*Apa*I) SNP a trend towards association with IGFBP1 levels in the parent-of-origin specific association analysis ($p$=0.04 and $p$=0.09, respectively). Interestingly, even though no significant or suggestive linkages were observed, the relation with IGFBP1 levels was visible in the linkage analysis. In

**Table 5.** Results of the parent-of-origin specific association analysis using the paternally inherited allele only.

| | | 6815_6819delAGGGC | | | 1156T>C | | | 820G>A (*Apa*1) | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Trait | α | wt | del | *p* | T | C | *p* | G | A | *p* |
| n | | 420 | 92 | | 311 | 171 | | 392 | 103 | |
| Age (yrs) | | 25.2 (24.7-25.6) | 25.1 (24.1-26.0) | | 25.4 (24.9-26.0) | 24.9 (24.2-25.6) | | 25.2 (24.8-25.7) | 24.6 (23.7-25.5) | |
| Gestational age (wks) | | 37.1 (36.9-37.4) | 37.0 (36.5-37.5) | | 36.9 (36.6-37.2) | 37.4 (37.0-37.8) | | 37.2 (36.9-37.4) | 36.8 (36.3-37.2) | |
| Birth weight (g) | 0.025 | 2555 (2510-2600) | 2549 (2458-2640) | 0.90 | 2550 (2499-2602) | 2563 (2493-2632) | 0.78 | 2537 (2493-2582) | 2606 (2523-2689) | 0.15 |
| Body height (cm) | 0.025 | 172 (171-173) | 171 (170-173) | 0.35 | 172 (171-173) | 172 (170-173) | 0.36 | 172 (171-173) | 172 (171-174) | 0.85 |
| Body mass (kg) | 0.01 | 64.2 (63.2-65.2) | 64.8 (62.7-66.9) | 0.59 | 64.8 (63.6-66.0) | 65.2 (63.7-66.8) | 0.66 | 64.4 (63.4-65.4) | 65.2 (63.3-67.2) | 0.47 |
| BMI ($kg/m^2$)[a] | 0.01 | 21.7 (21.3-22.0) | 21.8 (21.2-22.5) | 0.64 | 21.7 (21.3-22.1) | 21.9 (21.4-22.4) | 0.48 | 21.7 (21.3-22.0) | 21.8 (21.2-22.5) | 0.61 |
| Fat mass (kg)[a] | 0.01 | 13.9 (13.4-14.5) | 14.0 (12.9-15.1) | 0.90 | 13.7 (13.1-14.4) | 14.7 (13.8-15.6) | 0.08 | 13.8 (13.3-14.4) | 14.2 (13.1-15.3) | 0.58 |
| Lean body mass (kg) | 0.01 | 49.4 (48.9-50.0) | 49.9 (48.8-51.1) | 0.42 | 50.1 (49.4-50.7) | 49.9 (49.0-50.7) | 0.71 | 49.6 (49.1-50.2) | 49.8 (48.7-50.9) | 0.76 |
| S4SF (mm)[a] | 0.01 | 43.3 (41.4-45.2) | 43.0 (39.3-47.0) | 0.90 | 43.3 (41.2-45.6) | 44.8 (41.9-47.9) | 0.42 | 42.5 (40.6-44.5) | 45.7 (41.9-49.8) | 0.14 |
| WHR (%) | 0.05 | 77.4 (76.9-77.9) | 78.0 (77.0-79.1) | 0.29 | 77.4 (76.8-78.1) | 77.8 (77.0-78.6) | 0.49 | 77.3 (76.7-77.9) | 78.0 (77.0-79.1) | 0.20 |
| IGFBP1 (ng/ml)[a] | 0.025 | 14.5 (13.4-15.5) | 12.2 (10.5-14.2) | 0.04 | 13.2 (12.2-14.4) | 16.0 (14.4-17.9) | **0.005** | 14.9 (13.8-16.0) | 13.0 (11.3-15.0) | 0.09 |
| Fasting glucose (mmol/l) | 0.025 | 4.72 (4.67-4.76) | 4.71 (4.62-4.80) | 0.91 | 4.73 (4.68-4.78) | 4.74 (4.67-4.80) | 0.90 | 4.69 (4.64-4.73) | 4.79 (4.70-4.88) | 0.04 |
| Fasting insulin (pmol/l)[a] | 0.025 | 35.3 (33.8-36.9) | 35.9 (32.6-39.4) | 0.76 | 35.3 (33.5-37.3) | 34.8 (32.3-37.4) | 0.74 | 34.9 (33.3-36.6) | 37.0 (33.8-40.5) | 0.25 |
| Leptin (ng/ml)[a] | 0.025 | 4.34 (4.06-4.63) | 4.88 (4.27-5.57) | 0.11 | 4.45 (4.13-4.81) | 4.54 (4.10-5.02) | 0.77 | 4.19 (3.91-4.48) | 4.70 (4.11-5.37) | 0.13 |
| Total cholesterol (mmol/l) | 0.025 | 4.91 (4.80-5.01) | 5.24 (5.03-5.45) | **0.004** | 5.01 (4.89-5.13) | 4.97 (4.81-5.13) | 0.67 | 4.94 (4.83-5.05) | 5.02 (4.81-5.23) | 0.49 |
| LDL-cholesterol (mmol/l) | 0.025 | 2.89 (2.80-2.99) | 3.18 (2.99-3.37) | **0.007** | 2.97 (2.86-3.08) | 2.97 (2.82-3.11) | 0.98 | 2.92 (2.82-3.02) | 2.99 (2.79-3.18) | 0.55 |
| HDL-cholesterol (mmol/l) | 0.05 | 1.60 (1.56-1.65) | 1.65 (1.56-1.74) | 0.38 | 1.62 (1.57-1.67) | 1.57 (1.50-1.63) | 0.21 | 1.60 (1.55-1.64) | 1.60 (1.52-1.69) | 0.87 |
| Triglycerides (mmol/l)[a] | 0.05 | 0.85 (0.81-0.89) | 0.90 (0.82-0.99) | 0.26 | 0.86 (0.82-0.91) | 0.86 (0.80-0.93) | 0.97 | 0.86 (0.83-0.91) | 0.87 (0.79-0.95) | 0.95 |
| NEFA (mmol/l)[a] | 0.05 | 0.55 (0.53-0.58) | 0.53 (0.49-0.57) | 0.26 | 0.54 (0.52-0.57) | 0.56 (0.52-0.60) | 0.44 | 0.55 (0.53-0.57) | 0.53 (0.49-0.58) | 0.48 |

Data are expressed as least squares mean (95% confidence interval). [a]Geometric least squares mean (95% confidence interval (CI)). α = significance threshold. BMI = body mass index, IGFBP1 = insulin-like growth factor protein 1, n = number of individuals, NEFA = non-esterified fatty acids, S4SF = sum of four skinfolds, WHR = waist-to-hip ratio.

the present study, the fit of the conventional and the full imprinting model was compared using the AIC (Table 4). In case no effect is present, the conventional linkage model will always be preferred above the full imprinting model (which is translated in a lower AIC), simply because the conventional linkage model uses 1 *df* less. However, IGFBP1 was the only phenotype for which the full imprinting model had a better fit (lower AIC) compared to the conventional linkage model, attributable to the paternal chromosome. Nevertheless, the QTL effect was not significant, probably due to the small sample size of the linkage study (108 DZ twins).

Taken together, this study provides evidence that common variants in *IGF2* determine serum IGFBP1 levels. IGFBP1 is a member of the IGFBP family that modulates activity, bio-availability and tissue distribution of IGF1 and IGF2, of which IGF1 is besides insulin the most powerful peptide with a glucose lowering effect.[40,41] In total there are six IGFBPs, but IGFBP1 is acutely down-regulated by insulin and is suggested to be the short-term regulator of IGF1 bioactivity.[42-44] Low IGFBP1 levels predict the development of abnormal glucose regulation and have been associated with an unfavourable cardiovascular risk profile.[45,46] Conversely, in patients with acute myocardial infarction and type 2 diabetes, high IGFBP1 levels have been associated with an increased risk of cardiovascular mortality and morbidity, which is probably the result of hepatic insulin resistance in these patients.[47] Since circulating IGFBP1 is generally considered to be of hepatic origin, the effect observed in the present study is possibly the result of a modified *IGFBP1* expression mediated by IGF2 in the liver. This is in agreement with Lee et al.[48] who demonstrated in human hepatoma cells that IGF2 is able to suppress *IGFBP1* expression by binding to the IGF1 receptor (IGF1R).[48] Although *IGF1R* expression levels are low in the adult liver,[49] IGF2 can also bind to the insulin receptor that is abundantly expressed in the adult liver.[50,51] Interestingly, in the present study the association with IGFBP1 levels was only significant in the parent-of-origin specific analysis. This is surprising since in the adult liver *IGF2* is primarily transcribed from the bi-allelicly active P1 promoter.[4] However, in the adult liver *IGF2* is also transcribed, although to a lower extent, from the paternally active P0, P2, P3 and P4 promoters.[2,4,52] Accordingly, the total expression level of the paternal *IGF2* allele in the liver is higher than the expression level of the maternal *IGF2* allele, which is probably translated in the detected parent-of-origin specific association. On the other hand, fasting IGFBP1 levels are inversely correlated with fasting insulin levels and positively correlated with insulin sensitivity.[46,53] Therefore the observed association with IGFBP1 levels might also be a result of an improved whole body glucose uptake possibly mediated by locally produced IGF2 in for instance muscle, where only the paternally inherited *IGF2* allele is expressed.[2]

The variants examined in the present study have previously been associated with adult body height in a study of Heude et al.[29] They observed that the minor alleles of the 6815_6819delAGGGC and the 820G>A (*Apa*I) SNP were associated with shorter body height while the minor allele of the 1156T>C SNP was associated with taller body height (see also Table 6).[29] This is striking because in our study the minor allele of the 6815_6819delAGGGC and the 820G>A (*Apa*I) SNP were associated with lower IGFBP1 levels while the minor allele of the 1156T>C SNP was associated with higher IGFBP1 levels. Since IGFBP1 levels are inversely correlated with IGF1 levels,[53] one would expect that subjects having lower IGFBP1 levels, thus having more free IGF1, would be taller and the other way around, but this is apparently not the case. Concerning the large number of tests performed in the present study, our results could reflect random associations by chance. However LD is low among

**Table 6.** Reported associations of Heude et al.[29] and Gaunt et al.[28] between the 6815_6819delAGGGC, the 1156T>C and the 820G>A (ApaI) SNPs with body height and BMI, respectively.

| SNP | Study | Trait | | S | Genotype | | | *p* | $p_{All}$ |
|---|---|---|---|---|---|---|---|---|---|
| 6815_6819delAGGGC | | | | | wtwt | wtdel | deldel | | |
| | Heude et al.[29] | Body height | $N_M$ | | 1229 | 765 | 117 | | |
| | | | Mean (SEM) | M | 174.7 (0.18) | 174.2 (0.23) | 174.8 (0.58) | 0.23 | |
| | | | $N_W$ | | 1514 | 958 | 188 | | |
| | | | Mean (SEM) | W | 161.7 (0.15) | 161.4 (0.19) | 160.9 (0.43) | 0.058 | 0.027 |
| | Gaunt et al.[28] | BMI | $N_M$ | | 1377 | 906 | 111 | | |
| | | | Mean (SD) | M | 26.6 (3.5) | 26.2 (3.4) | 25.3 (3.6) | 0.00012 | |
| 1156 T/C | | | | | TT | TC | CC | | |
| | Heude et al.[29] | Body height | $N_M$ | | 829 | 1000 | 330 | | |
| | | | Mean (SEM) | M | 174.3 (0.22) | 174.6 (0.20) | 174.8 (0.35) | 0.37 | |
| | | | $N_W$ | | 1091 | 1217 | 412 | | |
| | | | Mean (SEM) | W | 161.3 (0.18) | 161.4 (0.17) | 162.5 (0.29) | 0.002 | 0.001 |
| | Gaunt et al.[28] | BMI | $N_M$ | | 403 | 812 | 352 | | |
| | | | Mean (SD) | M | 26.2 | 26.3 (3.5) | 26.8 (3.6) | 0.017 | |
| 820G>A (*Apa*1) | | | | | GG | GA | AA | | |
| | Heude et al.[29] | Body height | $N_M$ | | 1079 | 827 | 149 | | |
| | | | Mean (SEM) | M | 174.8 (0.19) | 174.2 (0.22) | 174.4 (0.52) | 0.068 | |
| | | | $N_W$ | | 1316 | 1054 | 209 | | |
| | | | Mean (SEM) | W | 161.8 (0.16) | 161.5 (0.18) | 160.9 (0.41) | 0.07 | 0.009 |
| | Gaunt et al.[28] | BMI | $N_M$ | | 1362 | 1019 | 179 | | |
| | | | Mean (SD) | M | 26.4 (3.3) | 26.2 (3.4) | 25.3 (3.2) | 0.0004 | |

M = men-only, $N_M$ = number of men, $N_W$ = number of women, $p_{All}$ = *p*-value calculated incorporating data of both men and women in the model, S = sample, SD = standard deviation, SEM = standard error of the mean, W = women-only.

the three variants (max $r^2$=0.22), and the effect of the 6815_6819delAGGGC and the 820G>A (*Apa*I) SNP was in the opposite direction of the effect of the 1156T>C SNP; a pattern that is in accordance with the literature (see also Table 6).[28,29] Consequently, it is unlikely that our results reflect false positive associations. Moreover, in contrast to umbilical cord IGFBP1 levels that are inversely correlated with fetal growth,[54] in our sample adult IGFBP1 levels do not correlate with adult body height, but are inversely correlated with fasting insulin and fasting glucose levels (data not shown), which is in agreement with the literature.[46,53] This indicates that adult IGFBP1 is a marker for insulin sensitivity instead of a marker for adult body height. Hence, improved insulin sensitivity (thus higher IGFBP1 levels) in the major allele carriers of the 6815_6819delAGGGC and the 820G>A (*Apa*I) SNP, and in the minor allele carriers of the 1156T>C SNP might result in an increased insulin-mediated growth, possibly translated in a slightly higher body height as reported by Heude et al.[29] In addition, Gaunt et al.[28] observed that major allele carriers of the 6815_6819delAGGGC and the 820G>A (*Apa*I) SNP, and minor allele carriers of the 1156T>C SNP had a higher BMI (see Table 6). This observation might be explained by the stimulating effect of insulin on lipid storage in adipocytes, as hypothesised by Prudente et al.[55] who argued that genetic determinants improving insulin sensitivity might have an opposite unfavourable effect on body weight.

In the present study, the paternally inherited minor allele of the *IGF2* 6815_6819delAGGGC variant was significantly associated with higher total and LDL-cholesterol levels ($p$=0.004 and $p$=0.007, respectively). To our knowledge, the (negative) studies of 't Hart et al.[25] and O'Dell et al.[22], who studied the relation between the 820G>A (*Apa*I) SNP and triglycerides and cholesterol levels, are the only association studies published thus far that examined the relation between a polymorphism in *IGF2* and dyslipidaemia. However, our study is not the first study reporting a relation between *IGF2* and cardiovascular risk factors. A recently published study of Kadlecová et al.[15] reported that an insertion of 14 nucleotides in the *Igf*2 gene was associated with triglycerides levels in rats, and with cholesterol levels in male rats only. Nevertheless, we are the first study reporting an association between an *IGF2* SNP and dyslipidaemia in humans, and accordingly further studies are required to verify our findings.

In summary, this study did not replicate previously reported associations between the 6815_6819delAGGGC, the 1156T>C and the 820G>A (*Apa*I) SNPs, with body height, BMI and birth weight. However, in this study the paternally inherited alleles of these common variants in the *IGF2* gene showed association with IGFBP1 and cholesterol levels, indicating that *IGF2* may have a role in glucose homeostasis and the lipid metabolism.

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## 6.8 SUPPLEMENTARY MATERIAL

### *6.8.1 Phenotypes*

Zygosity was determined using sequential analysis based on sex, fetal membranes, umbilical cord blood groups, placental alkaline phosphatase and DNA marker analysis. Birth weights were obtained from obstetric records, and gestational age reported by the obstetrician was calculated as the number of completed weeks of pregnancy based on the last menstrual period. Between February 1997 and April 2000, the twins visited the research centre in Leuven for a 2 h examination, which started in the morning after an overnight fast. Participants were measured barefoot and lightly clothed. Standing height (cm) was measured to the nearest 0.1 cm with a Harpenden fixed stadiometer (Holtain, Crosswell, UK). Body mass (kg) was measured on a balance scale (SECA, Hamburg, Germany) to the nearest 0.1 kg. Body mass index (BMI) was calculated as body mass divided by the square of height (kg/m2). Waist and hip circumferences were measured with a flexible steel tape to 1 mm accuracy. Waist circumferences were taken at the smallest point between the costal margin and the iliac crest and hip circumference at the widest part of the hips, generally at the level of the greater trochanters. Waist-to-hip circumference ratio (WHR) was expressed as a percentage. Lean body mass was measured using a bioelectrical impedance analyzer (BIA310; Biodynamics, Seattle, WA, USA). Fat mass (kg) was calculated by subtracting the value for lean body mass from total body mass. Skinfold thicknesses were taken in duplicate, to 0.1 mm accuracy with a Harpenden skinfold calliper (British Indicators, St Albans, UK) at the biceps, triceps, subscapular and suprailiac. The four thicknesses were summed (S4SF) to evaluate the overall subcutaneous fatness. Blood samples were drawn to measure plasma hormone concentrations. Plasma leptin was measured with an immunoradiometric assay in a coated tube (Diagnostic Systems Laboratories, Webster, TX, USA). Plasma lipids (triglycerides, total cholesterol and HDL-cholesterol) were measured on an auto-analyser (AU600; Olympus, Kyoto, Japan). LDL-cholesterol was estimated using Friedewald's formula.[56] Non-esterified fatty acids (NEFA) were measured using a colorimetric assay with the optical density measured at 550 nmol/l. Plasma glucose was measured using the hexokinase method (Olympus AU600). Plasma insulin was determined using a microparticle enzyme immunoassay (Axsym; Abbott Laboratories, Chicago, IL, USA). Insulin-like growth factor binding protein 1 (IGFBP1) was measured by radio-immunoassay, as described.[57]

### *6.8.2 Multiple testing*

Since multiple tests were performed, a principal component factor analysis (PCFA) was carried out on all phenotypes to assess the number of separate hypotheses that were actually tested. The PCFA was performed on residuals adjusted for significant covariates and the number of factors was selected based on a minimal eigenvalue of 1.0. To produce interpretable factors we used an orthogonal Varimax rotation, since oblique rotation (i.e. Oblimin) showed that the inter-factor correlations were low (<0.10). In addition, the factor loadings yielded by oblique rotation were very similar to those produced by the Varimax rotation. Variables sharing at least 50% of their variance with a factor, equivalent to a factor

loading of >0.70, were considered as measuring the same construct and were included in the factor.
In total seven factors were extracted by PCFA (Table S2). Factor 1 contained lean body mass and four obesity parameters, respectively body mass, BMI, fat mass and S4SF. Factor 2 showed high loadings for total cholesterol and LDL-cholesterol. Factor 3 contained IGFBP1 and fasting glucose levels, and factor 4 fasting insulin and leptin levels. Factor 5 included birth weight and adult body height and factor 6 showed high loadings with HDL-cholesterol only. In factor 7 no traits had a factor loading >0.70 (Table S2).
Within each factor, the number of phenotypes ($n_v$) was summed and to determine the significance threshold for these phenotypes a Bonferroni correction was performed ($\alpha/n_v$, where $\alpha$=0.05). Consequently, the significance threshold applied in the association analysis for body mass, BMI, fat mass, lean body mass and S4SF was 0.01, and for total cholesterol, LDL-cholesterol, IGFBP1, fasting glucose, fasting insulin, leptin, birth weight and body height 0.025. The remaining phenotypes preserved the significance threshold of 0.05 (Table S2).

**Table S1.** Covariate(s) incorporated in the association analysis.

| Trait | Covariates[a] |
|---|---|
| Birth weight | Sex, gestational age and chorionicity |
| Body height | Sex and age |
| Body mass | Age and body height |
| BMI[b] | Age |
| Fat mass[b] | Sex, age and body height |
| Lean body mass | Sex, age and body height |
| S4SF[b] | Sex and age |
| WHR | Zygosity, sex and age |
| IGFBP1[b] | Zygosity, sex and BMI |
| Fasting glucose | Zygosity, sex and WHR |
| Fasting insulin[b] | Age and S4SF |
| Leptin[b] | Sex, age and S4SF |
| Total cholesterol | Age and S4SF |
| LDL-cholesterol | Sex, age and S4SF |
| HDL-cholesterol | Sex and WHR |
| Triglycerides[b] | Sex and S4SF |
| NEFA[b] | Sex and S4SF |

[a]Covariates were incorporated into the model, when the F-test indicated $p<0.10$. [b]Transformed to natural logarithmic scale. BMI = body mass index, IGFBP1 = insulin-like growth factor protein 1, NEFA = non-esterified fatty acids, S4SF = sum of four skinfolds, WHR = waist-to-hip ratio.

**Table S2.** Factor loadings of the pre-diabetic phenotypes.

| | Factors | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | F | α |
| Birth weight | -0.02 | 0.00 | 0.01 | 0.11 | **0.80** | 0.08 | -0.13 | 5 | 0.025 |
| Body height | 0.03 | -0.04 | -0.06 | -0.16 | **0.80** | -0.05 | 0.05 | 5 | 0.025 |
| Body mass | **0.97** | 0.00 | 0.05 | 0.04 | 0.08 | -0.03 | -0.06 | 1 | 0.01 |
| BMI | **0.96** | -0.01 | 0.06 | 0.05 | 0.04 | -0.01 | -0.07 | 1 | 0.01 |
| Fat mass | **0.85** | 0.03 | -0.09 | 0.10 | 0.04 | 0.08 | 0.08 | 1 | 0.01 |
| Lean body mass | **0.80** | 0.00 | 0.16 | 0.02 | 0.13 | -0.08 | -0.19 | 1 | 0.01 |
| S4SF | **0.87** | -0.04 | -0.03 | -0.10 | -0.05 | 0.02 | 0.05 | 1 | 0.01 |
| WHR | 0.65 | 0.06 | -0.06 | 0.06 | -0.24 | -0.01 | 0.02 | - | 0.05 |
| IGFBP1 | -0.01 | -0.05 | **-0.77** | 0.00 | 0.05 | 0.00 | 0.21 | 3 | 0.025 |
| Fasting glucose | 0.02 | -0.07 | **0.79** | 0.10 | 0.00 | 0.06 | 0.15 | 3 | 0.025 |
| Fasting insulin | 0.07 | 0.03 | 0.29 | **0.72** | 0.01 | -0.23 | 0.24 | 4 | 0.025 |
| Leptin | 0.06 | -0.02 | -0.08 | **0.86** | -0.07 | 0.13 | -0.24 | 4 | 0.025 |
| Total cholesterol | 0.01 | **0.98** | -0.05 | 0.05 | 0.00 | 0.14 | 0.06 | 2 | 0.025 |
| LDL-cholesterol | 0.02 | **0.95** | 0.03 | -0.05 | -0.05 | -0.07 | -0.09 | 2 | 0.025 |
| HDL-cholesterol | -0.01 | 0.27 | -0.08 | 0.11 | 0.05 | **0.84** | 0.23 | 6 | 0.05 |
| Triglycerides | -0.01 | 0.32 | -0.28 | 0.24 | 0.02 | -0.68 | 0.25 | - | 0.05 |
| NEFA | -0.08 | -0.03 | -0.04 | -0.06 | -0.08 | 0.05 | **0.88** | 7 | 0.05 |
| Total variance (%) | 4.43 | 2.05 | 1.44 | 1.40 | 1.39 | 1.29 | 1.17 | - | - |
| Cumulative variance (%) | 4.43 | 6.48 | 7.92 | 9.32 | 10.71 | 12.00 | 13.17 | - | - |

Factor loadings >0.70 are shown in **boldface type**. α = significance threshold, BMI = body mass index, F = factor, IGFBP1 = insulin-like growth factor protein 1, NEFA = non-esterified fatty acids, S4SF = sum of four skinfolds, WHR = waist-to-hip ratio.

**Table S3.** Genotype frequencies and numbers, and allele frequencies of the *IGF2* SNPs calculated using a sub-sample that only included one randomly selected twin per pair.

| | Homozygous major allele (n) | Heterozygous (n) | Homozygous minor allele (n) | Major allele | Minor allele |
|---|---|---|---|---|---|
| 6815_6819delAGGGC | 0.578 (178) | 0.360 (111) | 0.062 (19) | 0.76 | 0.24 |
| 1156T>C | 0.418 (122) | 0.428 (125) | 0.154 (45) | 0.63 | 0.37 |
| 820G>A (*Apa*I) | 0.515 (156) | 0.413 (125) | 0.073 (22) | 0.72 | 0.28 |

**Table S4.** Allele frequencies and numbers of the D11S4046 microsatellite marker.

| | Parents | | | Twins[a] | | |
|---|---|---|---|---|---|---|
| D11S4046 | Allele | n | $p_{HWE}$ | Allele | n | $p_{HWE}$ |
| 178 | - | - | | 0.001 | 2 | |
| 182 | 0.176 | 156 | | 0.173 | 188 | |
| 184 | 0.099 | 88 | | 0.092 | 116 | |
| 186 | 0.201 | 178 | | 0.208 | 259 | |
| 188 | 0.058 | 51 | | 0.061 | 73 | |
| 190 | 0.019 | 17 | | 0.016 | 16 | |
| 192 | 0.026 | 23 | | 0.024 | 31 | |
| 194 | 0.105 | 93 | | 0.095 | 114 | |
| 196 | 0.168 | 149 | | 0.179 | 221 | |
| 198 | 0.096 | 85 | | 0.095 | 127 | |
| 200 | 0.047 | 42 | | 0.052 | 64 | |
| 202 | 0.005 | 4 | 0.32 | 0.004 | 5 | 0.57 |

[a]Frequencies of the twins are calculated using a sub-sample, that only included one randomly selected twin per pair (n=353). n = number of alleles, $p_{HWE}$ = *p*-value of the Hardy-Weinberg equilibrium test.

**Table S5.** Results of the association analyses based on both parental alleles with the *IGF2* 6815_6819delAGGGC variant.

| | | 6815_6819delAGGGC | | | |
|---|---|---|---|---|---|
| Trait[a] | α | wtwt | wtdel | deldel | $p_G$ |
| n | | 347 | 218 | 37 | |
| Birth weight | 0.025 | 2557 (2510-2605) | 2540 (2482-2598) | 2626 (2492-2759) | 0.49 |
| Body height | 0.025 | 172 (171-173) | 172 (171-173) | 171 (168-173) | 0.52 |
| Body mass | 0.01 | 64.5 (63.5-65.6) | 64.3 (62.9-65.6) | 65.7 (62.5-68.9) | 0.71 |
| BMI[b] | 0.01 | 21.8 (21.4-22.1) | 21.7 (21.2-22.1) | 22.3 (21.3-23.4) | 0.54 |
| Fat mass[b] | 0.01 | 14.1 (13.5-14.7) | 13.7 (13.0-14.4) | 14.2 (12.6-16.1) | 0.59 |
| Lean body mass | 0.01 | 49.5 (48.8-50.1) | 49.7 (48.9-50.4) | 51.0 (49.2-52.8) | 0.27 |
| S4SF[b] | 0.01 | 44.1 (42.0-46.2) | 43.0 (40.6-45.5) | 44.6 (38.9-51.1) | 0.75 |
| WHR | 0.05 | 77.5 (76.9-78.0) | 77.5 (76.8-78.2) | 79.0 (77.3-80.6) | 0.22 |
| IGFBP1[b] | 0.025 | 14.4 (13.3-15.6) | 14.4 (13.0-15.9) | 11.4 (8.92-14.5) | 0.18 |
| Fasting glucose | 0.025 | 4.72 (4.67-4.77) | 4.72 (4.66-4.78) | 4.73 (4.59-4.88) | 0.98 |
| Fasting insulin[b] | 0.025 | 35.4 (33.7-37.2) | 35.7 (33.6-38.0) | 39.4 (33.9-45.7) | 0.42 |
| Leptin[b] | 0.025 | 4.33 (4.03-4.65) | 4.49 (4.11-4.91) | 4.83 (3.91-5.98) | 0.56 |
| Total cholesterol | 0.025 | 4.90 (4.79-5.01) | 5.06 (4.92-5.20) | 5.30 (4.98-5.63) | 0.03 |
| LDL-cholesterol | 0.025 | 2.89 (2.79-2.99) | 3.04 (2.91-3.16) | 3.19 (2.89-3.49) | 0.05 |
| HDL-cholesterol | 0.05 | 1.59 (1.54-1.64) | 1.61 (1.55-1.67) | 1.68 (1.55-1.82) | 0.42 |
| Triglycerides[b] | 0.05 | 0.87 (0.82-0.91) | 0.85 (0.80-0.91) | 0.90 (0.78-1.05) | 0.75 |
| NEFA[b] | 0.05 | 0.56 (0.54-0.59) | 0.56 (0.52-0.59) | 0.50 (0.43-0.57) | 0.20 |

Data are expressed as least squares (LS) mean (95% CI). [a]The units of the phenotypic characteristics and abbreviation definitions are presented in Table 5. [b]Geometric LSmean (95% CI). α=significance threshold, BMI = body mass index, IGFBP1 = insulin-like growth factor protein 1, NEFA = non-esterified fatty acids, $p_G$ = *p*-value general association model, S4SF = sum of four skinfolds, WHR = waist-to-hip ratio.

**Table S6.** Results of the association analyses based on both parental alleles with the *IGF2* 1156T>C SNP.

| | | 1156T>C | | | |
|---|---|---|---|---|---|
| Trait[a] | α | TT | TC | CC | $p_G$ |
| n | | 242 | 241 | 89 | |
| Birth weight | 0.025 | 2545 (2489-2602) | 2569 (2513-2625) | 2549 (2455-2642) | 0.81 |
| Body height | 0.025 | 172 (171-173) | 172 (171-173) | 172 (170-174) | 0.98 |
| Body mass | 0.01 | 64.9 (63.5-66.2) | 64.4 (63.1-65.7) | 65.1 (63.0-67.2) | 0.81 |
| BMI[b] | 0.01 | 21.8 (21.4-22.2) | 21.7 (21.3-22.1) | 21.9 (21.2-22.6) | 0.93 |
| Fat mass[b] | 0.01 | 13.8 (13.2-14.6) | 13.9 (13.2-14.6) | 15.1 (13.9-16.3) | 0.17 |
| Lean body mass | 0.01 | 50.1 (49.3-50.8) | 49.5 (48.8-50.3) | 49.2 (48.0-50.4) | 0.40 |
| S4SF[b] | 0.01 | 44.1 (41.7-46.7) | 42.7 (40.4-45.1) | 46.4 (42.4-50.8) | 0.25 |
| WHR | 0.05 | 77.4 (76.7-78.1) | 77.7 (77.0-78.4) | 77.5 (76.4-78.6) | 0.84 |
| IGFBP1[b] | 0.025 | 14.1 (12.9-15.5) | 14.0 (12.8-15.4) | 15.8 (13.6-18.2) | 0.38 |
| Fasting glucose | 0.025 | 4.72 (4.66-4.78) | 4.70 (4.65-4.76) | 4.77 (4.68-4.87) | 0.47 |
| Fasting insulin[b] | 0.025 | 35.2 (33.2-37.4) | 35.7 (33.6-37.8) | 35.2 (31.9-38.8) | 0.95 |
| Leptin[b] | 0.025 | 4.58 (4.20-5.00) | 4.26 (3.91-4.64) | 4.66 (4.06-5.36) | 0.36 |
| Total cholesterol | 0.025 | 4.94 (4.81-5.07) | 5.06 (4.93-5.19) | 4.89 (4.67-5.11) | 0.27 |
| LDL-cholesterol | 0.025 | 2.92 (2.80-3.04) | 3.02 (2.90-3.14) | 2.91 (2.71-3.11) | 0.40 |
| HDL-cholesterol | 0.05 | 1.60 (1.55-1.66) | 1.61 (1.56-1.67) | 1.55 (1.46-1.64) | 0.53 |
| Triglycerides[b] | 0.05 | 0.85 (0.80-0.90) | 0.89 (0.84-0.94) | 0.84 (0.76-0.93) | 0.47 |
| NEFA[b] | 0.05 | 0.53 (0.51-0.56) | 0.57 (0.54-0.60) | 0.58 (0.54-0.64) | 0.13 |

Data are expressed as LSmeans (95% CI). [a]The units of the phenotypic characteristics and abbreviation definitions are presented in Table 5. [b]Geometric LSmean (95% CI). α=significance threshold, BMI = body mass index, IGFBP1 = insulin-like growth factor protein 1, NEFA = non-esterified fatty acids, $p_G$ = *p*-value general association model, S4SF = sum of four skinfolds, WHR = waist-to-hip ratio.

**Table S7.** Results of the association analyses based on both parental alleles with the *IGF2* 820G>A SNP.

| | | 820G>A | | | |
|---|---|---|---|---|---|
| Trait[a] | α | GG | GA | AA | $p_G$ |
| n | | 313 | 233 | 45 | |
| Birth weight | 0.025 | 2557 (2507-2608) | 2537 (2480-2594) | 2638 (2512-2764) | 0.34 |
| Body height | 0.025 | 172 (171-173) | 172 (171-173) | 174 (172-176) | 0.19 |
| Body mass | 0.01 | 64.7 (63.5-65.8) | 64.1 (62.9-65.4) | 65.4 (62.3-68.4) | 0.69 |
| BMI[b] | 0.01 | 21.8 (21.4-22.1) | 21.7 (21.2-22.1) | 21.9 (20.9-22.9) | 0.85 |
| Fat mass[b] | 0.01 | 14.1 (13.5-14.7) | 13.7 (13.1-14.5) | 13.9 (12.4-15.7) | 0.72 |
| Lean body mass | 0.01 | 49.6 (49.0-50.3) | 49.4 (48.7-50.1) | 50.3 (48.7-52.0) | 0.58 |
| S4SF[b] | 0.01 | 43.5 (41.5-45.7) | 43.8 (41.4-46.3) | 44.3 (38.9-50.4) | 0.97 |
| WHR | 0.05 | 77.5 (76.9-78.1) | 77.8 (77.1-78.5) | 76.9 (75.3-78.5) | 0.53 |
| IGFBP1[b] | 0.025 | 14.5 (13.4-15.8) | 14.6 (13.2-16.0) | 12.1 (9.58-15.2) | 0.30 |
| Fasting glucose | 0.025 | 4.69 (4.64-4.74) | 4.73 (4.67-4.79) | 4.86 (4.72-5.00) | 0.06 |
| Fasting insulin[b] | 0.025 | 34.7 (32.9-36.5) | 36.1 (34.0-38.3) | 40.5 (35.1-46.8) | 0.11 |
| Leptin[b] | 0.025 | 4.31 (4.00-4.64) | 4.35 (3.99-4.74) | 5.04 (4.08-6.21) | 0.38 |
| Total cholesterol | 0.025 | 4.96 (4.84-5.08) | 5.02 (4.88-5.16) | 5.08 (4.76-5.41) | 0.71 |
| LDL-cholesterol | 0.025 | 2.97 (2.86-3.08) | 2.97 (2.84-3.09) | 3.01 (2.72-3.31) | 0.96 |
| HDL-cholesterol | 0.05 | 1.57 (1.52-1.62) | 1.65 (1.59-1.70) | 1.62 (1.49-1.76) | 0.11 |
| Triglycerides[b] | 0.05 | 0.89 (0.84-0.94) | 0.84 (0.79-0.89) | 0.84 (0.73-0.97) | 0.38 |
| NEFA[b] | 0.05 | 0.56 (0.53-0.58) | 0.57 (0.54-0.60) | 0.49 (0.43-0.56) | 0.13 |

Data are expressed as LSmeans (95% CI). [a]The units of the phenotypic characteristics and abbreviation definitions are presented in Table 5. [b]Geometric LSmean (95% CI). α=significance threshold, BMI = body mass index, IGFBP1 = insulin-like growth factor protein 1, NEFA = non-esterified fatty acids, $p_G$ = *p*-value general association model, S4SF = sum of four skinfolds, WHR = waist-to-hip ratio.

# CHAPTER 7

# GENERAL DISCUSSION

The aim of this thesis was to uncover genetic variants involved in type 2 diabetes (T2D) using a well-defined twin sample recruited from the East-Flanders Prospective Twin Survey (EFPTS). We applied a quantitative trait approach and used different kinds of genetic methodologies. In this chapter, the most important observations are highlighted and inconsistencies with the literature are evaluated. In addition, recent and future developments in the field of the genetics of T2D and twin research are discussed.

## 7.1 HERITABILITIES

In order to define the size of the genetic component underlying the quantitative traits studied in this thesis, heritability estimates were calculated using the classical twin approach (Chapter 2). In general, the majority of the variation of the traits related to obesity, glucose intolerance/insulin resistance and dyslipidaemia in the studied sample was explained by genetic factors. Compared to other twin studies, the heritability estimates in this Belgian twin sample were in a slightly higher range, probably attributable to the accurate zygosity determination,[1] young sample age and the homogeneous composition of the sample (Chapter 2).[2-4] High heritabilities are preferred, because the degree of heritability is an important determinant of the power to detect disease-related genes.[5]

## 7.2 LINKAGE

Subsequently, univariate variance components linkage analyses were performed using polymorphic microsatellite markers located in the close vicinity of eleven T2D candidate genes (Chapters 3 and 6). No significant linkages were observed (LOD>3), although some genes (*ABCC8*, *ADIPOQ*, *IGFBP1* and *LEP*) showed suggestive linkage with one or more T2D metabolic risk factors (LOD>1). These results were not completely unexpected, since for linkage only the DZ twins were informative ($n_{max}$=112 pairs).[6] Prior to analyses it was hypothesised that power could be gained by genotyping polymorphic microsatellite markers in the close vicinity of the candidate genes, but this was unfortunately not sufficient. Some candidate genes that were negative for linkage were also targeted in the SNP association study, where they did show significant association with one of the T2D related metabolic risk factors (*IGF2*, *LEPR* and *PPARγ*) (Chapters 4, 5 and 6). This was also to be expected because association is in general more powerful than linkage,[6] and for the association study almost three times more individuals were informative ($n_{max}$=612 individuals) than for the linkage study. Nevertheless, the results of the *LEP* and *LEPR* genes of the linkage and association analysis were quite remarkable. In the linkage study, *LEP* showed suggestive linkage with birth weight while the *LEPR* was negative for linkage (Chapter 3). In the association study, the SNP in *LEP* (19G>A) was significantly associated with HDL-cholesterol, and both the SNPs in the *LEPR* (K109R and Q223R) were strongly associated with birth weight (Chapter 5). These results seem inconsistent, however, linkage and association are completely different concepts. When a SNP is significantly associated with a certain trait, this implies that the tested SNP is causal or is in linkage disequilibrium (LD) with a causative polymorphism nearby. However, linkage with a microsatellite marker only implies that functional variants can be located in that candidate gene region. Moreover, when a gene is

involved in the regulation of a trait, there are many possible spots within that gene that may be changed and affect the trait.[7] Thus it is expected that multiple functional variants within a gene, although many will be rare, underlie a linkage signal. In addition, since linkage extends over a long distance it is also possible that multiple genes with multiple variants underlie a single linkage peak.[7] It is more likely that the linkage signals reported in this thesis are at least partly due to rare variants (with a larger effect size), because our linkage study lacks the power to identify common disease variants as demonstrated in Chapter 6. In this chapter three common polymorphisms in the *IGF2* gene showed association with IGFBP1 levels in the parent-of-origin specific association analysis. Despite the fact that in the parent-of-origin specific linkage analysis the full imprinting model for IGFBP1 levels gave a better fit than the conventional linkage model, linkage was neither significant nor suggestive. To conclude, the at first sight inconsistent results of the linkage and association analysis for the *LEP* and *LEPR* genes are not contradictory, but strongly indicate that leptin is involved in fetal growth.

For T2D and disease related traits many genome wide linkage scans have been performed and despite the fact that quite a lot of promising linkage peaks have been reported, thus far the follow up of these results has been disappointing.[8-12] The only well-established genetic risk factor for T2D identified by a linkage approach to date is the *TCF7L2* gene.[13] This slow progress is not surprising, since these quantitative trait loci (QTL) usually span large chromosomal regions that contain numerous genes.[8] In addition, several promising QTLs have not been replicated in other populations,[9-11] which supports the idea that besides common variants with relatively small effect sizes, also rare population specific variants with larger effect sizes are involved. The latter are believed to be of greater significance in targeted disease prevention and understanding of disease etiology.[14] Nevertheless, to identify these rare variants comprehensive sequencing of large numbers of individuals is required. Since it is yet too expensive to sequence those entire chromosomal regions, genes targeted for sequencing are often selected on the basis of prior knowledge. Although this is a logic strategy it might be misleading, given that the majority of the recently discovered T2D susceptibility genes using genome wide association were unsuspected genes (this will be discussed in one of the following paragraphs). However, sequencing technology is advancing rapidly and therefore functional variants underlying these QTLs are likely to be identified in the near future.[15]

## 7.3 ASSOCIATION

Next to linkage, association is another strategy to identify genetic factors underlying complex diseases. For T2D numerous genetic association studies have been carried out and as a consequence numerous positive and to a somewhat lesser extent negative associations have been reported, of which the latter is believed to be a result of publication bias.[16,17] To ascertain that positive associations represent true genotype-phenotype associations, replication in independent population samples is warranted. Usually, replication studies are performed using the straightforward case-control design. In this thesis, replication was carried out with a series of metabolic risk factors that predispose to or are associated with T2D, in order to extent current knowledge (Chapters 4, 5 and 6). To optimise the power of the design, potentially functional SNPs were selected that were either non-synonymous,

located in the promoter sequence or in the 5' and 3' untranslated region (UTR). Of these SNPs, some showed significant associations that were consistent with the literature. Some SNPs showed a significant association that appeared to be in the opposite direction than reported in the literature, and others showed no significant association. Unfortunately, this is a common feature in genetic association studies and represents a major problem for the ultimate identification of real disease modifying genetic variants.

A positive association can be explained in a number of ways: 1) The targeted SNP is the functional variant. 2) The targeted SNP is not the functional variant, but is in LD with the causal variant. 3) It results from population stratification caused by the occurrence of unknown subgroups in the study sample that differ in average trait value and allele frequencies. 4) It results from multiple testing (false positives).[16,18] Due to the increasing number of markers tested in genetic association studies, spurious associations as a result of multiple testing are currently a major issue in genetic epidemiology. To decrease the number of false positives, stringent significance thresholds and large samples sizes are required. It has been suggested that for replication studies very stringent significance thresholds may not be required.[19] However, our study was not designed as a traditional replication study and therefore we did need to control for multiple testing. The implemented correction for multiple testing may not have been stringent enough, but using a more stringent correction method would have increased the number of false negative results. To distinguish true associations from type I errors, positive associations were validated through literature study. In general, when the direction of the effect was in the same direction as reported in the literature, the association was considered a true association. However, when results appear inconsistent with the literature, one should be careful with definite conclusions. The *PPARγ* P12A SNP is a good example to illustrate this. The A allele of this SNP is generally considered as protective for T2D,[20] however, in this study we observed that the A allele was associated with increased obesity (Chapter 4). This seems in conflict with the literature, because obesity is positively associated with T2D risk and therefore one should expect that the A allele of the *PPARγ* P12A SNP would also be protective for obesity. However a large meta-analysis confirms our result,[21-24] and the A allele of the *PPARγ* P12A SNP can be considered as protective for T2D but as a risk factor for obesity. A possible explanation for this might be the presence of a second SNP in *PPARγ* (C1431T), given that the protective effect of the A allele disappears when the A allele is on the same chromosome as the T allele of the C1431T SNP that has been associated with an increased BMI, but not with insulin indices (demonstrating the additional information provided by haplotype analysis opposed to single SNP analysis).[25,26] On the other hand, this paradox is also observed in T2D patients receiving thiazolidinediones (*PPARγ* agonists). In these patients improved glycaemic control is positively correlated with weight gain, since these *PPARγ* agonists improve adipocyte function resulting in an increased capacity to store fat.[27,28] Whatever the underlying mechanism may be, the *PPARγ* P12A SNP clearly demonstrates the complexity of these metabolic disorders and shows that apparently conflicting results should not immediately be marked as false positive results. We also observed seemingly conflicting results with the three SNPs in the *IGF2* gene (Chapter 6). These SNPs have previously been associated with body height and BMI,[29,30] but we observed association with IGFBP1 levels. Since IGFBP1 levels are inversely correlated with IGF1 levels,[31] one would expect that subjects having low IGFBP1 levels are taller. In contrast, in the literature the genotype groups with the lowest IGFBP1 levels were the smallest and the leanest.[29,30] However, in our study, adult IGFBP1

levels did not correlate with adult body height, but were inversely correlated with fasting insulin levels, indicating that adult IGFBP1 might be a marker for insulin sensitivity instead of a marker for adult body height. Hence, we postulated that higher IGFBP1 levels possibly represent higher insulin sensitivity, resulting in an increased insulin-mediated growth and improved triglycerides storage in adipocytes. In contrast to the *PPARγ* P12A SNP, these SNPs in *IGF2* have not been extensively studied and additional research is necessary to confirm our results.

Inconsistent associations can also be due to differences in genetic background. LD patterns are considered to be quite similar among European populations.[32] Consequently, when a positive association is observed and the direction of the effect is in conflict with the literature, it is unlikely that the targeted SNP in the study population is in LD with a different common functional SNP having an opposite effect. However, the targeted SNP might be in LD with a rare causal population specific variant. Such an association will never be replicated in a different population and will be considered as false positive, although this rare variant might be of significance for disease prevention in that specific population. Unfortunately, extensive resequencing of the gene region is the only (expensive) method to find out whether such a positive association is true or false.

Also for lack of replication there are several explanations: 1) The initial result might have been a false positive as a result of population stratification or multiple testing. The latter occurs when multiple analyses are carried out, but only a small subset is reported.[33] 2) The replication study has inadequate power to replicate the initial report. 3) The lack of replication is due to real population differences (genetic background, environmental exposures etc).[34] According to the current criteria for sample sizes required for genetic association studies, the present study is considered to be underpowered. However, the majority of the SNPs examined in this study have previously been associated with T2D or disease related phenotypes, in studies with a similar or even a smaller sample size (see also supplementary tables S5–S15, Chapter 4). Therefore, in theory, the sample size in this thesis should have been sufficient to replicate most of these previously reported associations. However, it is important to note that T2D usually manifests itself at a more advanced age (the mean age of onset is 50 years).[35] The mean age of the studied sample in this thesis is only 25 years and at this young age metabolic abnormalities may only be moderately apparent. Moreover, the study sample is quite lean (mean BMI is only 22 $kg/m^2$), which might be due to the young age and/or a healthy lifestyle. Because T2D is a multifactorial disease, persons who are genetically predisposed will only develop T2D when they are exposed to specific environmental risk factors, e.g. an unhealthy lifestyle. Consequently, the young age of the studied sample and possibly their healthy lifestyle might somewhat reduce the power and therefore increase the probability of false negative findings. On the other hand, compared to other twin studies, the heritability estimates of the traits studied in this thesis are in a higher range (Chapter 2). This indicates that the phenotypic variation observed in our young twin sample is highly explained by genetic factors, which is a major advantage for genetic linkage and association analysis.

The most striking negative result in our study occurred with respect to the *KCNJ11* E23K SNP, which is currently considered to be a genuine genetic risk factor for T2D. However, *KCNJ11* is involved in insulin secretion and unfortunately in our study direct measurements on insulin secretion are unavailable.[36]

Another remarkable observation in our study is the lack of association between genetic variants and their corresponding protein serum levels. For instance, in our association study, the *LEP* 19G>A SNP was significantly associated with HDL-cholesterol levels, but not with leptin levels (Chapter 5). The same was observed in our linkage study, where *LEP* showed suggestive linkage with birth weight, but not with leptin levels. In addition, *IGFBP1* showed suggestive linkage with fat mass, fasting insulin and leptin levels, but not with IGFBP1 levels (Chapter 3). These results might be interpreted as false positives, because if the genetic variants would really be involved, effects on serum protein levels would also be expected. However, this is not necessarily true, it rather indicates that the genetic variant does not affect gene expression or translation in the tissue of origin, which is adipose tissue for leptin and liver for IGFBP1. To illustrate this, we refer to the study of Van Laere et al.[37], who identified a paternally expressed QTL affecting muscle growth, fat deposition and size of the heart in pigs. This QTL was explained by a substitution in intron 3 of the *IGF2* gene causing higher *IGF2* mRNA expression in postnatal cardiac and skeletal muscle, but not in liver. Since the liver is the primary source of IGF2, no association between this causal SNP and IGF2 serum levels was observed.[37]
Our results indicate that the quantitative trait approach is a powerful tool to increase the understanding of how genetic risk factors modulate metabolism. For instance, the availability of birth weight data enabled us to demonstrate that genetic variants in the *LEPR* regulate fetal growth, and to suggest a possible role for the *LEPR* in explaining the inverse relationship between birth weight and the development of adult metabolic disease. However, a pitfall of this approach is that due to the large number of tests performed a percentage of the positive associations observed will represent false positives.

## 7.4 GENOME WIDE ASSOCIATION STUDIES

The growing understanding of the variation in the human genome[38] together with recent advances in genotyping technology, have made it possible to genotype hundreds of thousands of SNPs simultaneously and to perform association studies on a genome wide scale. The first genome wide association (GWA) study for T2D was published in early 2007,[39] and since then many have followed including GWA studies for obesity and dyslipidaemia.[40-63]
Before the GWA studies were published only three genes had reproducibly been associated with T2D, namely *KCNJ11*, *PPARγ* and *TCF7L2*. The GWA studies for T2D have confirmed these findings and identified six novel genes: *CDKAL1*, *CDKN2A|2B*, *IGF2BP2, FTO, SLC30A8* and *HHEX*.[39-44] Furthermore, in a recently published meta-analysis of three GWA studies, six additional T2D genes were identified: *JAZF1*, *CDC123/CAMK1D*, *TSPAN8/LGR5*, *THADA*, *ADAMTS9* and *NOTCH2/ADAM30*.[46] For obesity, the most promising genes identified by GWA studies thus far are *CTNNBL1*, *FTO*, *INSIG2*, *MC4R* and *PFKP*, but the results are less consistent than the results of the GWA studies for T2D, probably due to differences in study design and smaller sample sizes.[50-54,64]

### *7.4.1 Power and multiple testing*

Besides the *KCNJ11* and *PPARγ* genes, none of the candidate genes studied in this thesis have been identified by these GWA studies for T2D and obesity. This does however not

imply that the other candidate genes studied in this thesis are futile candidates; although GWA is a powerful tool to detect new disease related genes, there are some limitations that must be taken into account. A major limitation of GWA is that due to the huge number of loci tested, a very stringent significance threshold is required to restrict the number of false positive results. When a genotyping platform comprising 500,000 SNPs is used, *p*-values have to be lower than approximately $5*10^{-7}$ to supply robust statistical evidence for association.[65] To obtain such low *p*-values extremely large sample sizes are required. In addition, it should be kept in mind that GWA is based on indirect association, which requires even more power than is needed for direct association studies. Consequently, due to the stringent significance threshold required, the GWA studies performed for T2D and obesity have thus far generated a lot of false negative results. This can be nicely illustrated by the E23K *KCNJ11* and P12A *PPARγ* SNPs. Associations with these two SNPs and T2D were not strong in any of the initial GWA studies. Only after pooling data of three GWA studies, eventually comprising 10,829 cases and 12,622 controls, the *p*-value for *KCNJ11* and *PPARγ* were $6.7*10^{-11}$ and $1.7*10^{-6}$, respectively.[40,42,43] Because a significant role of these SNPs in T2D had been established before, a lot of effort has been put into confirming these SNPs in these GWA studies. However, the *p*-values of the *KCNJ11* and *PPARγ* SNPs in the separate scans were not impressive and numerous variants will have had similar or even lower *p*-values, but were considered as non-significant and no effort has been taken (yet) to confirm or exclude them.

### *7.4.2 Selection criteria cases and controls*

Although, in this thesis, a quantitative trait approach was used and problems associated with defining cases and controls are not applicable to our study, it is of interest to discuss the impact of the selection criteria used to classify the cases and controls on the outcomes of the GWA studies. In the GWA study for T2D of the Wellcome Trust Case Control Consortium the second most significant association was observed for a polymorphism in the *FTO* gene ($p=2.0*10^{-8}$).[42] Strikingly, the *FTO* gene was not identified by other GWA studies for T2D.[39-41,43] It appears that this gene contributes to an increased T2D risk by modulating BMI and since other GWA studies had matched their cases and controls for BMI or selected lean T2D cases, they were unable to detect this variant.[39,40,66] The involvement of *FTO* in obesity has independently been confirmed by GWA studies for obesity.[51,53] The *FTO* gene clearly demonstrates that the criteria used to select cases and controls have a huge impact on the study outcome. Interestingly, it has been demonstrated that, besides *FTO* and *PPARγ*, the majority of the risk loci identified for T2D are probably involved in pancreatic ß-cell function (*CDC123/CAMK1D, CDKAL1*, *CDKN2A|2B*, *HHEX*, *IGF2BP2*, *JAZF1*, *KCNJ11*, *SLC30A8*, *TCF7L2* and *TSPAN8/LGR5*).[41,65,67-71] This is remarkable because T2D is believed to be the final result of both insulin resistance and ß-cell dysfunction. Because obesity is highly correlated with insulin resistance, it has been suggested that variants contributing to insulin resistance would be better detectable in an obesogenic background.[72] It has also been argued that insulin resistance is not as heritable as insulin secretion and that fewer variants, or variants with a more modest effect size and/or that rare variants might be involved in insulin resistance.[72] In addition, it has been put forward that the insulin sensitivity measurements used might only poorly represent insulin resistance at the tissue level.[72] Others have suggested that the results of these GWA studies indicate that the genetic susceptibility for

T2D is due to genetic factors involved in ß-cell function rather than insulin action.[65] However, one should keep in mind that T2D is a heterogeneous disease, preceded by a pre-diabetic stage that includes subjects with impaired fasting glucose (IFG), impaired glucose tolerance (IGT) or both (combined glucose intolerance, CGI).[73] Subjects with isolated IFG have elevated fasting glucose levels but show a normal response during an oral glucose tolerance test (OGTT), while subjects with isolated IGT have normal fasting glucose levels but show an abnormal response during OGTT (elevated 2h-glucose). In addition, subjects with CGI have a combination of IGT and IFG (for a detailed overview see reference [73]). In both IFG and IGT, ß-cell dysfunction is present which is characterised by an impaired early-phase insulin response, and subjects with IGT also have an impaired late-phase insulin response.[73] However, it is believed that people with IFG and IGT differ with respect to the site of insulin resistance, where subjects with IFG are considered to have predominantly hepatic insulin resistance and subjects with IGT to mainly have muscle insulin insensitivity.[73] Since these distinct states eventually result in severe hyperglycaemia and thus the diagnosis of T2D, it is likely that the cases used in genetic association studies for T2D represent a mixture of subjects that were IGT, IFG or CGI in their pre-diabetic stage. Thus these T2D cases differ in sites of insulin resistance but all have an impaired early-phase insulin secretion, and as a consequence these association studies have probably more power to identify genes involved in ß-cell function than genes involved in insulin resistance. To identify insulin resistance genes, it might be more powerful to use subjects with isolated IGF or IGT, which possibly enables the identification of genes involved in hepatic or muscle insulin sensitivity, respectively. Besides the selection of the cases, the criteria used to classify the controls are also questionable. For instance, in the GWA study for T2D of Sladek et al.[39] controls were classified on the basis of fasting glucose levels. As described above, subjects with isolated IGT have normal fasting glucose levels and would according to this criterion be classified as controls. Moreover, insulin resistant subjects with sufficient β-cell compensation also have normal fasting glucose levels. Hence, such a selection criterion can reduce the power to identify genes involved in insulin resistance. These problems can be avoided by analysing appropriate quantitative measurements instead of defining cases and controls.

### *7.4.3 Gender influences*

The majority of the GWA studies for T2D and obesity published thus far did not take sex-specific effects into account, despite that there are huge metabolic differences between men and women. For instance, men are taller, heavier, have less fat mass, more lean body mass and have a different body fat distribution compared to women. In addition, women have lower fasting glucose levels and higher fasting insulin, leptin and HDL-cholesterol levels.[74] Moreover, it has been demonstrated that genes are differently expressed among men and women.[75] As has been demonstrated in this thesis (Chapter 4), if sex-specific effects are not taken into consideration and a genetic risk factor has an effect in one sex only, performing association analyses in the complete sample can diminish this sex-specific effect and might result in a non-significant *p*-value. On the other hand, when a significant association is observed in the complete sample this effect might be the result of a sex-specific association and consequently this genetic risk factor is only a therapeutic target in one of the sexes. Therefore, studying sex-specific effects is likely to improve our understanding of the genetics

of metabolic disorders and will have important implications in the future for the development of genotype-based medicine.
Nevertheless, GWA studies have proven to be a powerful tool in identifying new genes involved in complex diseases and will provide further great new insights on the pathogenesis of T2D. However, there are still many drawbacks in the current analyses to be overcome and it is yet too early to exclude or confirm the candidate genes studied in this thesis.

## 7.5 TWIN STUDIES AND THEIR FUTURE PERSPECTIVES

To unravel the genetics of human disease, the twin design is a very popular design, because it is suitable for a range of quantitative and molecular genetic analyses. For instance, to quantify the relative contribution of genetic and environmental factors to the variation of a phenotype of interest, twin studies are considered to offer the most valid estimations.[76] Accordingly, for numerous complex disorders and conditions twin studies have been used to demonstrate that genetic predisposition plays a significant role. It has been demonstrated that poor fetal growth is associated with an increased risk for T2D and cardiovascular disease.[77-79] MZ MC twins suffer a more adverse intrauterine environment than MZ DC twins, which results in a lower intra-pair birth weight correlation for MZ MC twins compared to MZ DC twins. Since the classical twin design assumes equal prenatal environment for MZ MC, MZ DC and DZ twins, it has been argued that the twin design is an unreliable method to estimate heritabilities for metabolic phenotypes related to T2D and cardiovascular disease.[80] For the metabolic risk factors studied in this thesis no significant differences between the MZ MC and MZ DC twin correlations were observed, indicating that the classical twin design is a valid method to estimate heritabilities of these phenotypes (Chapter 2). Nevertheless, failure to detect a significant difference due to low power cannot be excluded.
However, quantitative genetic analyses provide no information about the underlying molecular mechanism and therefore several gene mapping techniques have been developed, including linkage and association. Since DZ twins are matched for age and for a broad range of pre- and postnatal factors, they are perfect for sib-pair based linkage analysis.[76] Although, MZ twins are uninformative for linkage, in this thesis it was hypothesised that incorporating the phenotypic data of the MZ twins would improve the variance components linkage analysis, because the variance components would be estimated more accurately. Univariate variance components linkage analyses were performed with and without incorporating the phenotypic data of the MZ twins for a series of T2D related metabolic risk factors (Chapter 3). The two methods gave fairly similar linkage results, except for birth weight where the QTL effect was underestimated when phenotypic data of the MZ MC twins were incorporated. Although the sample size and the number of microsatellite markers tested were limited, these results indicated that in our study sample, including phenotypic data of MZ twins did not significantly improve the variance components linkage analysis. Moreover, if chorionicity is an important confounder and information on chorionicity is missing, phenotypic data of MZ twins should not be included in linkage analysis.
Although linkage studies have been very successful in mapping rare high-risk disease alleles involved in single gene disorders, they have been much less successful in mapping common disease variants with small effects. Consequently, to unravel the genetics of complex diseases, the more powerful association studies are recommended. The twin design is also

suitable for these association studies. Moreover, the use of MZ twins reduces the genotyping costs with 50%, since one genotype is associated with two phenotypes. However, as association analysis does not require related individuals, the majority of the studies performed use unrelated individuals who are easier to recruit.

In addition to linkage and association, gene expression profiling using microarray technology, which allows measuring the expression levels of thousands of genes simultaneously, is another commonly used approach to identify disease related genes. By comparing genome-wide expression profiles of "healthy" and "diseased" tissue samples, molecular biological pathways and/or genes involved in the disease of interest can be identified.[81] For these gene expression studies, MZ twins are of special interest. Studying MZ twins discordant for a certain disease or condition allows to control for genomic sequence variation and for a range of environmental factors,[82] which strongly improves the power of a gene expression study.[83] However, for these gene expression studies the effect of chorionicity should be assessed. In this thesis no significant differences in MZ MC and MZ DC twin correlations were observed, but the studied phenotypes are complex multifactorial traits. This is in contrast to gene expression phenotypes that are likely to be less complex traits. If indeed due to an adverse intrauterine environment metabolic adaptations occur that are maintained throughout live, chorionicity might confound gene expression profiles. The more adverse intrauterine environment of MZ MC twins might result in larger intra-pair differences of certain gene expression phenotypes for MZ MC twins compared to MZ DC twins. Therefore, it is of great interest to compare intra-pair correlations of gene expression phenotypes for MZ MC and MZ DC twins. Genes showing a lower intra-pair correlation for MZ MC compared to MZ DC twins are candidates for the fetal programming hypothesis.

However, besides genetics there is an additional level of complexity in the process of transcribing and translating DNA code to its end product, which is called epigenetics. Epigenetics refers to changes in gene functioning that occur without changes in DNA sequence and are mitotically, and sometimes meiotically heritable. In a multicellular organism the genome is identical among different cells, but the epigenome varies between tissues resulting in cell-type-specific regulation of gene expression by several epigenetic mechanisms including DNA methylation, histone modification and non-coding RNA-mediated silencing.[84] Epigenetic regulation of gene expression might account for the difficulties to uncover the causal genes involved in complex diseases, like T2D. Until recently, studies examining the relation between epigenetic modifications and human disease have generally relied on investigating epigenetic status at specific candidate genes. However, because of recent technological developments it has become possible to use unbiased approaches that profile epigenetic features such as DNA methylation patterns and histone modifications on a genome-wide scale.[85] To study the contribution of epigenetic modifications in the establishment of complex phenotypes in human, MZ twins are simply the perfect model. In contrast to their genome, which is identical, their epigenomes differ.[86] The comparison of epigenetic features like DNA methylation patterns in MZ twins discordant for a certain phenotype will provide more insight in how epigenetic modifications relate to certain metabolic states.

In conclusion, in the past twin studies have been a valuable tool for providing evidence that genetic susceptibility contributes to the development of numerous disorders. For gene mapping purposes, the DZ twins are perfectly suited for sib-pair-based linkage analysis and both MZ and DZ twins can be used for genetic association analysis. In addition, MZ twins are

powerful subjects for gene expression studies and will in the future be essential to understand the significance of epigenetic alterations to the modification of complex disease risk.

## 7.6 REFERENCES


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# SUMMARY
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# SUMMARY

Type 2 diabetes (T2D), which accounts for approximately 90-95% of all diabetes patients, is the final result of insufficiencies in both insulin action (insulin resistance) and secretion. The worldwide incidence of T2D is increasing at an alarming rate and the disease is currently considered to be one of the major causes of premature illness and death. This worldwide increase is strongly correlated with the dramatically rising incidence of obesity, which is characterised by excess accumulation of adipose tissue, secreting a variety of active molecules, including proinflammatory cytokines, non-esterified fatty acids (NEFA) and hormones that are believed to induce insulin resistance. Moreover, obesity, insulin resistance and T2D are accompanied with an abnormal lipid profile, including elevated triglycerides and decreased HDL-cholesterol levels that are associated with an increased cardiovascular risk. Epidemiological studies have demonstrated that factors related to the western lifestyle such as high calorie diets, high intake of saturated fat and reduced physical inactivity are strongly associated with T2D. However, besides the key role of these lifestyle factors, there is also clear evidence that genetic susceptibility contributes to the development of T2D. Currently, much research is focused on the genetics of T2D, in order to get a better understanding of the pathogenesis and eventually to achieve better, more personalised diagnostics, treatment and prevention.

In this thesis a candidate gene approach was used to identify genetic variants underlying quantitative traits that predispose to or are associated with T2D, that have been previously determined in a young twin sample recruited from the East Flanders Prospective Twin Study (EFPTS). The traits studied include parameters quantifying obesity, insulin sensitivity, dyslipidaemia as well as birth weight, since epidemiological studies have shown that children born small for gestational age are at increased risk for T2D.

The starting point in the search for genes is to estimate the degree of heritability of the disease related traits in the studied sample. Heritability is the proportion of phenotypic variation of a trait that can be attributed to genetic variation, and is an important determinant of the power to detect and localise disease-related genes. Although heritabilities can in principle be estimated from all kinds of related individuals, twin studies allow splitting up the total variation of a trait into genetic, shared environmental and unique environmental components, thus offering one of the most valid estimations. However, monozygotic monochorionic (MZ MC) twins suffer a more adverse intrauterine environment than MZ dichorionic (DC) twins, resulting in a lower intra-pair birth weight correlation for MZ MC twins as compared to MZ DC twins. Since poor fetal growth is associated with an increased risk for T2D and the classical twin study assumes equal prenatal environment for MZ MC, MZ DC and DZ twins, it has been argued that the classical twin study is an unreliable method to estimate heritabilities for phenotypes related to T2D. In **Chapter 2**, heritabilities were estimated for 18 quantitative traits that predispose to or are associated with T2D, measured in 138 MZ MC, 102 MZ DC and 138 DZ twin pairs. For these 18 phenotypes no significant differences between the MZ MC and MZ DC twin correlations were observed, which indicates that the classical twin design is a valid method to estimate heritabilities of these phenotypes. For body mass, BMI and fat mass we observed that men had higher heritability estimates than women, namely heritability estimates being 84, 85 and 81% in men and 74, 75 and 70% in

women, respectively. For waist-to-hip ratio (WHR), sum of four skinfolds (S4SF) and lean body mass heritability estimates were 70, 74 and 81%, respectively. The heritability estimates of fasting glucose, fasting insulin, homeostasis model assessments of insulin resistance and beta cell function, and insulin-like growth factor binding protein 1 (IGFBP1) levels were 67, 49, 48, 62 and 47%, respectively. In addition, the variation of total cholesterol, LDL-cholesterol, HDL-cholesterol, triglycerides, non-esterified fatty acids (NEFA) and leptin levels were for respectively 75, 78, 76, 58, 37 and 53% explained by genetic factors. Accordingly, total variation of traits related to obesity, insulin sensitivity and dyslipidaemia in this Belgian twin sample were highly explained by genetic factors.

In order to explain some of the genetic variance observed, we carried out univariate variance components linkage analysis in **Chapter 3.** Linkage was assessed by genotyping microsatellite markers located within or near functional candidate genes (encoding proteins with a suspected role in the pathophysiology of T2D), including the ATP-binding cassette sub-family C member 8 (*ABCC8*), adiponectin (*ADIPOQ*), glucokinase (*GCK*), insulin-like growth factor 1 (*IGF1*), IGF binding protein 1 (*IGFBP1*), insulin receptor (*INSR*), leptin (*LEP*), leptin receptor (*LEPR*), peroxisome proliferative activated receptor gamma (*PPARγ*) and the resistin (*RETN*) gene. Suggestive linkages (LOD>1) were observed between the *ABCC8* marker and WHR and HDL-cholesterol levels. Both markers flanking the *ADIPOQ* gene showed suggestive linkage with triglycerides levels, the upstream marker also with body mass and HDL-cholesterol levels. The *IGFBP1* marker showed suggestive linkage with fat mass, fasting insulin and leptin levels and the *LEP* marker showed suggestive linkage with birth weight. This study suggests that DNA variants in the *ABCC8*, *ADIPOQ*, *IGFBP1* and *LEP* gene region may predispose to T2D. In addition, we compared two methods when performing univariate single point variance components linkage analysis. 1) The standard method, comprising only phenotypic and genotypic data of 112 DZ twin pairs. 2) An extended method, also incorporating the phenotypic data of the 240 MZ twin pairs, which results in a more accurate estimation of the variance components as compared to the standard method. In general, the two methods yielded similar results. This was however not the case for birth weight, since LOD scores for *LEP* were considerably lower when the phenotypic data of MZ MC twins were incorporated. This suggests that including phenotypic data of MZ MC twins results in a reduced power to detect a quantitative trait locus for traits where chorionicity is an important confounder.

In addition to linkage we also carried out the more powerful genetic association analyses. To optimise the power of our design, we selected potentially functional single nucleotide polymorphisms (SNPs) that were either non-synonymous, located in the promoter sequence or located in the 5' or 3' untranslated region (UTR). For the genetic association analyses phenotypic and genotypic data were available for 396 MZ and 232 DZ twins.
In **Chapter 4**, we carried out a large association study with eleven SNPs (in eleven genes) that have previously been associated with T2D and/or disease related phenotypes. Association analyses were carried out with birth weight and parameters quantifying obesity, insulin sensitivity and dyslipidaemia measured at adult age, on the complete sample and for men and women separately. We observed that the A allele of the P12A SNP in the peroxisome proliferator activated receptor-γ gene (*PPARγ*), which is a transcription factor involved in adipocyte differentiation, lipid storage and glucose metabolism, was in the

complete sample associated with a higher weight at birth and a higher WHR. The minor allele of the -174G>C SNP in the gene encoding the proinflammatory cytokine interleukin-6 (*IL6*) was in the complete sample associated with higher HDL-cholesterol levels, in the men-only sample with higher NEFA levels and in the women-only sample with larger S4SF and lower triglycerides levels. In the glutamate decarboxylase 2 gene (*GAD2*), encoding the glutamic acid decarboxylase enzyme (GAD65) that catalyses the formation of the neurotransmitter γ-aminobutyric acid (GABA), which has been suggested to regulate food intake and inhibit glucose-induced insulin release, we genotyped the –243A>G SNP. We observed that heterozygote carriers of the –243A>G *GAD2* SNP had in the complete sample significantly lower fasting insulin levels than either homozygote carriers or non-carriers. In the men only sample, the G allele of the –243A>G *GAD2* SNP was significantly associated with lower fasting insulin levels and lower lean body mass. For the R72T SNP in the *PYY* gene, that encodes the food intake suppressing gut hormone peptide YY, we observed that R allele carrying women had lower fasting glucose levels. In addition, the Q allele of K121Q SNP in the ectoenzyme nucleotide pyrophosphate phosphodiesterase 1 gene (*ENPP1*), also known as plasma cell membrane glycoprotein 1 (*PC-1*) that impairs insulin signalling by interacting with the insulin receptor, was associated with lower NEFA levels in men and higher HDL-cholesterol levels in women. In the uncoupling protein 2 (*UCP2*), belonging to the mitochondrial inner membrane carrier protein family that diminishes ATP production by causing a proton leak, the A allele of the -866G>A SNP was in women associated with higher LDL-cholesterol levels. Finally, the T allele of the P129T SNP in the gene encoding fatty acid amide hydrolase (*FAAH*) that inactivates the feeding behaviour stimulating endogenous cannabinoid receptor ligands, was in men associated with a higher weight at birth. No significant associations were observed with SNPs genotyped in the genes encoding the potassium inwardly-rectifying channel, subfamily J, member 11 (*KCNJ11*, E23K), angiotensin II type 1 receptor (*AGTR1*, A1166C), cytochrome P450 1A1 (*CYP1A1*, 6235T>C) and the insulin receptor substrate 2 (*IRS2*, G1057D). In summary, seven out of eleven SNPs studied in **Chapter 4** were associated with one or more metabolic risk factors. Most SNPs acted sex-specific, and if confirmed in other populations these sex-specific associations may be important in the development of future genotype-based medicine.

In **Chapter 5** we primarily focused on leptin, which is a satiety signal that inhibits food intake and stimulates energy expenditure by acting on the hypothalamus. Although leptin is primarily released by adipocytes and serum levels are highly correlated with adipose tissue mass, the hormone and its receptor are also expressed in other tissues, including pancreatic beta cells where it inhibits insulin secretion. During pregnancy leptin is produced by maternal and fetal adipose tissue as well as by the placenta, and in contrast to maternal leptin levels, umbilical cord leptin levels are positively correlated with birth weight. Since children born small for gestational age are at increased risk of developing T2D in adulthood, we hypothesised that leptin might be a possible candidate for the "fetal insulin hypothesis", which postulates that birth weight and T2D have common genetic antecedents. Therefore we examined in **Chapter 5** whether common SNPs in the leptin (*LEP*; 19G>A) gene and its receptor (*LEPR*; Q223R and K109R) are associated with birth weight and adult metabolic risk factors that predispose to or are associated with T2D. We observed that both coding SNPs in the *LEPR* (K109R and Q223R) were strongly associated with birth weight; subjects carrying the R allele of the *LEPR* K109R and/or the Q223R SNP had a significantly higher weight at

birth. In addition, we observed that G allele carriers of the *LEP* 19G>A SNP had significantly higher HDL-cholesterol levels. Although umbilical cord leptin levels are positively correlated with birth weight, there is currently no consensus on the significance of this correlation; whether it implies that leptin acts as a growth-promoting signal during fetal development or whether it is just a good marker for fetal adiposity. Our results, however, indicate that leptin does have a biologic function during prenatal growth, and suggests a possible role for the *LEPR* in explaining the inverse relationship between birth weight and the development of metabolic diseases in adulthood. In addition, our results indicate that the *LEP* 19G>A SNP might be protective against cardiovascular disease.

In **Chapter 6** we focused on the insulin-like growth factor 2 (*IGF2*) gene. *IGF2* is maternally imprinted and paternally expressed, and plays a key role during fetal development. After birth IGF2 is transcribed at a lower level, but several studies suggest that IGF2 has some postnatal metabolic effects as well. In **Chapter 6** three SNPs (6815_6819delAGGGC, 1156T>C and 820G>A (*Apa*I)) and a microsatellite marker in the close vicinity of *IGF2* were genotyped in the twins and their parents and we carried out conventional and parent-of-origin specific linkage and association analyses with birth weight, adult body height and parameters quantifying obesity, insulin sensitivity and dyslipidaemia. No significant or suggestive linkages were observed in either the conventional or in the parent-of-origin specific linkage analysis. In addition, we did not replicate previously reported associations between the studied SNPs and adult body height, BMI and birth weight. However, we did observe a very interesting association with IGFBP1 levels in the parent-of-origin specific association analysis, where carriers of the paternally inherited minor allele of the 1156T>C SNP had significantly higher IGFBP1 levels compared to carriers of the paternally inherited major allele. In addition, for the 6815_6819delAGGGC and the 820G>A (*Apa*I) SNP we observed a trend towards association with IGFBP1 levels in the parent-of-origin specific association analysis. Since, IGFBP1 levels are positively correlated with insulin sensitivity, our results indicate that *IGF2* might play a role in glucose homeostasis. In addition, we observed that the paternally inherited minor allele of the *IGF2* 6815_6819delAGGGC variant was significantly associated with higher total and LDL-cholesterol levels. Although a possible role for *IGF2* in the lipid metabolism has already been suggested by a study in rats, we are the first study reporting an association between an *IGF2* SNP and the lipid metabolism in humans.

# SAMENVATTING

Type 2 diabetes (T2D) is de meest voorkomende vorm van diabetes (90-95%) en wordt veroorzaakt door een combinatie van verminderde insuline gevoeligheid van de lichaamscellen (insuline resistentie) en een ontoereikende insuline afgifte door de alvleesklier. Wereldwijd neemt het aantal T2D patiënten drastisch toe en de aandoening wordt momenteel gezien als een van de belangrijkste oorzaken van vroegtijdige ziekte en sterfte. De wereldwijde toename van T2D is sterk gerelateerd aan het stijgend aantal mensen die lijden aan obesitas (zwaarlijvigheid). Obesitas wordt gekenmerkt door een abnormaal overschot aan vetweefsel dat allerlei actieve stoffen afscheidt, zoals pro-inflammatoire cytokines, vrije vetzuren en hormonen, die betrokken zijn bij het ontstaan van insuline resistentie. Bovendien gaan obesitas, insuline resistentie en T2D gepaard met een afwijkend lipidenprofiel (dyslipidemie), zoals hoge triglyceriden- en lage HDL-cholesterolgehaltes, dat het risico op hart- en vaatziekten verhoogt. Epidemiologische studies hebben aangetoond dat onze westerse levensstijl, die gekenmerkt wordt door overvloedig en calorierijk eten en een gebrek aan lichaamsbeweging, de belangrijkste oorzaak is van de drastische toename van het aantal T2D patiënten. Echter naast de sleutelrol van een ongezonde levenswijze, is er ook bewijs dat "genetische gevoeligheid" een belangrijke rol speelt bij het ontstaan van T2D. Het identificeren van de genetische factoren is een belangrijke strategie om de onderliggende moleculaire mechanismen die leiden tot het ontstaan van T2D te ontrafelen. Deze kennis zal leiden tot nieuwe aangrijpingspunten voor therapieën en op de lange termijn tot geïndividualiseerde behandelingen gebaseerd op genetische aanleg.

Om meer inzicht te krijgen in de genetische factoren die een rol spelen bij het ontstaan van T2D, werd in dit proefschrift gekeken naar de relatie tussen kandidaat-genen en kwantitatieve kenmerken die voorafgaan of gerelateerd zijn aan T2D in een populatie jong volwassen tweelingen die behoren tot het Oost-Vlaams Meerlingenregister. Naast parameters gemeten op volwassen leeftijd (±25 jaar) die lichaamssamenstelling, insuline gevoeligheid en dyslipidemie kwantificeren, is er ook gekeken naar geboortegewicht, aangezien uit onderzoek is gebleken dat kinderen met een laag geboortegewicht een verhoogd risico hebben op het krijgen van T2D.

De zoektocht naar genen begint met het schatten van de heritabiliteiten van de ziekte-gerelateerde kenmerken in de studiepopulatie. De heritabiliteit geeft de mate aan waarin verschillen in een bepaald kenmerk tussen individuen verklaard worden door genetische verschillen. Door de heritabiliteit van een kenmerk te bepalen kan worden nagegaan of een zoektocht naar onderliggende genetische factoren überhaupt wel zinvol is. Monozygote (MZ) tweelingen zijn genetisch identiek, maar hebben ook een deel van hun omgeving gemeenschappelijk. Verschillen tussen MZ tweelingen worden alleen toegeschreven aan unieke omgevingseffecten. Dizygote (DZ) tweelingen delen ook deels hun omgeving, maar hebben slechts 50% van hun genoom gemeenschappelijk. Verschillen tussen DZ tweelingen kunnen zowel worden toegeschreven aan genetische factoren als aan unieke omgevingsinvloeden. Door de informatie van MZ en DZ tweelingen te combineren (klassiek tweelingen design) kan de variatie van een bepaald kenmerk worden opgesplitst in variatie ten gevolge van genetische factoren (heritabiliteit), gedeelde omgevingsfactoren en unieke

omgevingsfactoren. Echter MZ tweelingen kunnen worden onderverdeeld in monochoriale (MC) en dichoriale (DC) tweelingen, waarvan de MZ MC tweelingen in een ongunstiger prenataal milieu verkeren dat vertaald wordt in een lagere intra-paar correlatie voor geboortegewicht dan de MZ DC tweelingen. Aangezien een ongunstige prenatale omgeving geassocieerd is met een verhoogd risico op T2D en het klassieke tweelingen design veronderstelt dat de prenatale omgeving gelijk is voor MZ MC, MZ DC en DZ tweelingen, heeft men geopperd dat het klassieke tweelingen design een onbetrouwbare methode is om heritabiliteiten te schatten van kenmerken die gerelateerd zijn aan T2D. In **Hoofdstuk 2** van dit proefschrift hebben wij heritabiliteiten bepaald van 18 kwantitatieve kenmerken die voorafgaan of gerelateerd zijn aan T2D, gemeten in 138 MZ MC, 102 MZ DC en 138 DZ tweelingenparen op volwassen leeftijd. Voor deze 18 kenmerken vonden wij geen significante verschillen tussen de intra-paar correlaties van MZ MC en MZ DC tweelingen, wat aangeeft dat het klassieke tweelingen design een valide methode is om heritabiliteiten van deze fenotypes te schatten. Voor de kenmerken lichaamsgewicht, body-mass index (BMI) en vetmassa vonden wij dat mannen een hogere heritabiliteit hadden dan vrouwen, namelijk de heritabiliteiten van deze kenmerken waren respectievelijk 84, 85 en 81% in mannen en 74, 75 en 70% in vrouwen. Schattingen van de heritabiliteit van de middel-heupomtrek-verhouding, de som van vier huidplooidikten en vetvrije massa betroffen 70, 74 en 81%. Bloedlipidengehaltes, waaronder totaal cholesterol, LDL-cholesterol, HDL-cholesterol, triglyceriden en vrije vetzuren hadden een heritabiliteit van respectievelijk 75, 78, 76, 58 en 37%. Tenslotte waren de heritabiliteiten van glucose, insuline, β-cell functie (HOMA), insuline resistentie (HOMA) en IGFBP1 gehaltes 67, 49, 48, 62 en 47%, respectievelijk. Deze resultaten tonen aan dat verschillen in kwantitatieve kenmerken die voorafgaan of gerelateerd zijn aan T2D in deze populatie jong volwassen tweelingen voor een groot gedeelte verklaard worden door genetische factoren.

Om een gedeelte van deze genetische variatie te verklaren hebben wij in **Hoofdstuk 3** koppelingsanalyses uitgevoerd met polymorfe microsatelliet markers (repeterende stukjes DNA die in lengte variëren, welke allelen genoemd worden) gelegen nabij kandidaat-genen die een rol spelen in insuline secretie, resistentie en obesitas en wellicht variaties bevatten die van invloed zijn op sommige van de T2D gerelateerde kenmerken. Koppelingsanalyse is erop gebaseerd dat wanneer een genetische marker gelokaliseerd is nabij een variant die van invloed is op bijvoorbeeld cholesterol, dan zullen DZ tweelingen die hetzelfde stukje DNA van hun ouders hebben geërfd een meer gelijke cholesterolspiegel hebben dan DZ tweelingen die verschillende stukjes DNA van hun ouders geërfd hebben. In totaal hebben we 10 kandidaat-genen bestudeerd, waaronder ATP-binding cassette sub-family C member 8 (*ABCC8*), adiponectine (*ADIPOQ*), glucokinase (*GCK*), insulin-like growth factor 1 (*IGF1*), IGF binding protein 1 (*IGFBP1*), insuline receptor (*INSR*), leptine (*LEP*), leptine receptor (*LEPR*), peroxisome proliferative activated receptor gamma (*PPARγ*) en resistine (*RETN*). Vijf van de twaalf genetische markers gelegen nabij vier kandidaat-genen gaven een positief signaal (LOD>1), waaronder de marker vlakbij *ABCC8* die koppeling vertoonde met de middel-heupomtrek-verhouding en HDL-cholesterol. De twee markers gelegen rondom het *ADIPOQ* gen vertoonde koppeling met het triglyceridengehalte, en de voorafgaand gelegen marker ook met lichaamsgewicht en HDL-cholesterol. De marker gelegen nabij *IGFBP1* vertoonde koppeling met vetmassa, insuline en leptine, en de *LEP* marker met geboortegewicht. Deze resultaten impliceren dat in of nabij *ABCC8*, *ADIPOQ*, *IGFBP1* en *LEP* mogelijk genetische

varianten liggen die van invloed zijn op de positief bevonden kenmerken. Daarnaast hebben we ook nog twee koppelingsanalyse-methoden met elkaar vergeleken: 1) De standaard methode, waarin enkel gebruik werd gemaakt van de fenotypische en genotypische data van de 112 DZ tweelingenparen. 2) Een alternatieve methode, waarin ook de fenotypische data van de 240 MZ tweelingen in de analyse werd opgenomen, omdat dit mogelijk zou leiden tot een betere schatting van de variantie ten gevolge van genetische- en omgevingsfactoren. Over het algemeen gaven deze twee methoden min of meer gelijke resultaten. Dit was echter niet het geval voor geboortegewicht, aangezien de signalen voor *LEP* aanmerkelijk lager waren wanneer de fenotypische data van de MZ MC tweelingen in de koppelingsanalyse was opgenomen. Dit suggereert dat wanneer choriontype een belangrijke confounder (verstorende variabele) is, het opnemen van de fenotypische data van MZ MC tweelingen in de koppelingsanalyse zal resulteren in een lagere statistische kracht.

Naast koppelingsanalyse hebben we ook nog een andere methode toegepast om genetische varianten op te sporen die betrokken zijn bij het ontstaan van T2D, namelijk associatieanalyse. Associatieanalyse verschilt van koppelingsanalyse, omdat bij koppelingsanalyse gekeken wordt naar overerving van stukjes DNA en een bepaald kenmerk binnen families, terwijl in associatiestudies gekeken wordt op populatieniveau. In associatiestudies worden meestal wijzigingen in de genetische code van één enkele nucleotide – oftewel single nucleotide polymorphisms (SNPs) – bestudeerd, die twee allelen hebben (bv allel A en B) en drie mogelijke genotypes (AA, AB en BB). In een associatieanalyse worden dan groepen individuen met verschillende genotypes met elkaar vergeleken om te kijken of een bepaald kenmerk (bv. cholesterolgehaltes) verschilt tussen deze groepen. In dit proefschrift hebben we enkel SNPs bestudeerd die een aminozuurverandering tot gevolg hebben of gepositioneerd zijn in regio's die van belang zijn voor transcriptie of translatie, waardoor deze SNPs mogelijk zouden kunnen leiden tot een verandering in de activiteit of in de hoeveelheid van het genproduct. Voor de associatieanalyses hadden wij genotypes en fenotypes beschikbaar van 396 MZ tweelingen en 232 DZ tweelingen. In **Hoofdstuk 4** hebben wij een grote associatiestudie uitgevoerd met elf SNPs (in elf verschillende genen) die in andere studies al eens positief bevonden waren met een verhoogd risico op T2D en/of ziekte gerelateerde kenmerken. In dit hoofdstuk hebben wij de relatie bestudeerd tussen deze elf SNPs en metabole risicofactoren voor T2D, in het gehele sample en voor mannen en vrouwen apart. Wij vonden dat het A allel van de P12A SNP in het peroxisome proliferative activated receptor gamma (*PPARγ*) gen dat betrokken is bij vetcelontwikkeling en vetopslag, in het gehele sample geassocieerd was met een hoger geboortegewicht en een hogere middel-heupomtrek-verhouding. Het C allel van de –174G>C SNP in het gen dat codeert voor het pro-inflammatoire cytokine interleukin-6 (*IL6*), was in het gehele sample geassocieerd met hogere HDL-cholesterolgehaltes, in de mannen met hogere vrije vetzuren gehaltes en in de vrouwen met een hogere som van vier huidplooidikten en lagere triglyceridengehaltes. In het glutamate decarboxylase 2 gen (*GAD2*), dat codeert voor het glutamic acid decarboxylase enzym (GAD65) dat de formatie katalyseert van neurotransmitter γ-aminobutyric acid (GABA), welke een rol zou spelen in de regulatie van voedselinname en een remmend effect zou hebben op de insulinesecretie, hebben we de –243A>G SNP gegenotypeerd. In het gehele sample hadden de heterozygote dragers (AG) van de –243A>G SNP lagere insulinegehaltes dan de homozygoten (AA en GG). Bovendien hadden mannelijke dragers van het G allel van de –243A>G SNP lagere

insulinegehaltes en een lagere vetvrije massa. De R72T SNP in het *PYY* gen, dat codeert voor het eetlustremmende darmhormoon peptide YY, was in vrouwen geassocieerd met lagere glucose gehaltes. Bovendien vonden we dat het Q allel van de K121Q SNP in het ectoenzyme nucleotide pyrophosphate phosphodiesterase 1 gen (*ENPP1*), dat de insuline signalering verstoort, geassocieerd was met lagere gehaltes vrije vetzuren in de mannen en met hogere HDL-cholesterolgehaltes in de vrouwen. Daarnaast vonden wij dat vrouwen die het A allel droegen van de -866G>A SNP in het uncoupling protein 2 (*UCP2*) gen, dat door mitochondriële ontkoppeling te veroorzaken energie verloren laat gaan als warmte, hogere LDL-cholesterolgehaltes hadden. Tenslotte was het T allel van de P129T SNP in het fatty acid amide hydrolase (*FAAH*) gen dat eetluststimulerende endocannabinoiden inactiveert, in mannen geassocieerd met een hoger geboortegewicht. Geen significante associaties werden gevonden met SNPs in genen die coderen voor de potassium inwardly-rectifying channel, subfamily J, member 11 (*KCNJ11*, E23K), angiotensin II type 1 receptor (*AGTR1*, A1166C), cytochrome P450 1A1 (*CYP1A1*, 6235T>C) en insulin receptor substrate 2 (*IRS2*, G1057D). Samengevat, zeven van de elf SNPs waren significant geassocieerd met een of meerdere T2D gerelateerde risicofactoren, waarvan de meeste associaties geslachtsspecifiek waren.

In **Hoofdstuk 5** hebben we ons primair gericht op het verzadigingshormoon leptine dat een signaal afgeeft aan de hersenen, waardoor de behoefte aan voedsel afneemt en het energieverbruik toeneemt. Ofschoon leptine grotendeels door de vetcellen wordt geproduceerd en de gehaltes in het bloed gelijk oplopen met de hoeveelheid vetmassa, komen het hormoon en zijn receptor ook in andere weefsels tot expressie, zoals in de alvleesklier waar het de insulinesecretie afremt. Tijdens de zwangerschap wordt leptine geproduceerd door zowel de vetcellen van de moeder als van de foetus, maar ook door de placenta, en de leptinegehaltes in het navelstrengbloed staan in directe relatie met de hoogte van het geboortegewicht. Aangezien kinderen die te klein geboren worden een verhoogd risico hebben op het krijgen van T2D, hebben wij gesteld dat leptine een mogelijke kandidaat is voor de "fetal insulin hypothesis", waarin beweerd wordt dat geboortegewicht en T2D bepaald worden door gemeenschappelijke genetische factoren. In **Hoofdstuk 5** hebben wij gekeken naar SNPs in het leptine gen (*LEP*; 19G>A) en zijn receptor (*LEPR*; K109R en Q223R) en hun relatie met geboortegewicht en metabole risicofactoren voor T2D gemeten op volwassen leeftijd. Wij vonden een sterke associatie tussen beide SNPs in het *LEPR* (K109R en Q223R) gen en geboortegewicht, namelijk individuen die drager waren van het R allel van de *LEPR* K109R en/of Q223R SNP hadden een significant hoger geboortegewicht. Bovendien vonden we dat dragers van het G allel van de *LEP* 19G>A SNP hogere HDL-cholesterolgehaltes hadden. Hoewel leptinegehaltes in het navelstrengbloed positief gerelateerd zijn aan geboortegewicht, is er momenteel geen eensgezindheid over de betekenis van deze samenhang. Sommigen beweren dat deze samenhang betekent dat leptine zich gedraagt als een groeibevorderend hormoon tijdens de foetale ontwikkeling, terwijl anderen stellen dat het impliceert dat leptine een goede marker is voor de hoeveelheid vetmassa in de foetus. Echter onze resultaten suggereren dat leptine wel degelijk een biologische functie heeft tijdens de prenatale ontwikkeling, en dat het *LEPR* gen een mogelijke genetische factor is die de relatie tussen laag geboortegewicht en verhoogd risico op T2D helpt verklaren. Bovendien suggereren onze resultaten dat het G allel van de *LEP* 19G>A SNP mogelijk beschermt tegen hart- en vaatziekten.

Tenslotte hebben we in **Hoofdstuk 6** het insulin-like growth factor 2 (*IGF2*) gen bestudeerd. Dit gen is maternaal "imprinted" (ingeprent) en komt alleen paternaal tot expressie. Dit betekent dat alleen de kopie van het *IGF2* gen afkomstig van de vader tot expressie komt, terwijl de kopie afkomstig van de moeder op non-actief staat. Het is bekend dat IGF2 een belangrijke rol speelt tijdens de prenatale ontwikkeling en een aantal onderzoeken suggereren dat IGF2 na de geboorte een mogelijke rol speelt in het glucose- en vetmetabolisme. In **Hoofdstuk 6** hebben wij drie varianten (6815_6819delAGGGC, 1156T>C SNP en 820G>A (*Apa*I) SNP) gelegen in het *IGF2* gen en een microsatelliet marker gesitueerd nabij het *IGF2* gen gegenotypeerd in de tweelingen en hun ouders. Vervolgens hebben wij standaard en "parent-of-origin" specifieke koppelings- en associatieanalyses uitgevoerd om te kijken of er een verband was tussen deze genetische varianten en geboortegewicht, lichaamslengte en T2D gerelateerde risicofactoren gemeten op volwassen leeftijd. In de "parent-of-origin" specifieke analyse werd alleen het allel dat afkomstig was van de vader in de koppelings- en associatieanalyse opgenomen. Wij vonden geen koppeling tussen de kenmerken en de microsatelliet marker in de standaard of in de "parent-of-origin" specifieke koppelingsanalyse. Bovendien konden wij de bevindingen van eerdere studies die een relatie vonden tussen deze genetische varianten en lichaamslengte, BMI en geboortegewicht niet bevestigen. Echter wij vonden wel een hele interessante relatie tussen de 1156T>C SNP en IGFBP1-gehaltes in de "parent-of-origin" specifieke associatieanalyse, waar individuen die het C allel van hun vader geërfd hadden significant hogere IGFBP1-gehaltes hadden dan individuen die het T allel van hun vader geërfd hadden. Bovendien vonden wij voor de andere twee SNPs een trend tot associatie met IGFBP1-gehaltes in de "parent-of-origin" specifieke associatieanalyse. Aangezien IGFBP1-gehaltes positief gecorreleerd zijn met insulinegevoeligheid, impliceren onze resultaten dat varianten in *IGF2* mogelijk een rol spelen in het glucosemetabolisme. Daarnaast vonden wij dat individuen die de 5 basenparen deletie 6815_6819delAGGGC van hun vader geërfd hadden significant hogere totale en LDL-cholesterolgehaltes hadden. Hoewel een mogelijke rol voor IGF2 in het lipidenmetabolisme al eerder was gesuggereerd in een studie met ratten, is onze studie de eerste die een relatie tussen een *IGF2* SNP en het lipidenmetabolisme in mensen rapporteert.

# Dankwoord

# DANKWOORD

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Nicole

# Curriculum Vitae

## CURRICULUM VITAE

Nicole Yvonne Paulus Souren werd geboren op 12 juli 1981 te Simpelveld. Van 1993 tot 1999 doorliep zij het Voorbereidend Wetenschappelijk Onderwijs (VWO) aan het College Sancta Maria te Kerkrade-West, waarna zij in september 1999 begon met de vijfjarige studie Zoötechniek (Dierwetenschappen) aan de universiteit van Wageningen. Nadat ze haar grote afstudeervak bij de vakgroep Adaptatiefysiologie met als onderwerp "post-partum oestrus gedrag in zeugen" had afgerond, vertrok zij voor 3 maanden op buitenlandse stage. Tijdens deze stage aan de Universität für Bodenkultur in Wenen werd haar interesse voor genetica gewekt. Terug in Nederland begon zij aan haar tweede afstudeervak bij de leerstoelgroep Fokkerij en Genetica, waar zij mocht werken aan een genetisch defect in vleeskuikens. Tijdens dit afstudeervak maakte zij voor het eerst kennis met moleculair genetische technieken en werd dankzij de uitstekende begeleiding enthousiast gemaakt voor het genetisch onderzoek. Ze behaalde haar universitair diploma in november 2004 en begon diezelfde maand aan een promotietraject als Assistent in Opleiding (AIO) aan de Universiteit Maastricht bij de afdeling Population Genetics, Genomics & Bioinformatics binnen de capaciteitsgroep Genetica en Celbiologie, verbonden aan het onderzoeksinstituut Nutrition and Toxicology Research Institute Maastricht (NUTRIM). Het promotieonderzoek was gericht op Type 2 Diabetes en had als doel om meer inzicht te krijgen in de genetische factoren die een rol spelen bij het ontstaan van Type 2 Diabetes. In dit onderzoek werd de relatie bestudeerd tussen kandidaat-genen en kwantitatieve kenmerken die voorafgaan of gerelateerd zijn aan Type 2 Diabetes in een populatie jong volwassen tweelingen die behoren tot het Oost-Vlaams Meerlingenregister. Vanaf 1 november 2008 is ze werkzaam als postdoc op de afdeling Complex Genetica aan de Universiteit Maastricht, waar ze haar werkzaamheden in het tweelingonderzoek voortzet.

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